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Generation of sinapinic acid-containing extracts from Irish rapeseed meal and characterisation of their bioactive properties for potential human health applications

A thesis submitted for the degree of

Doctor of Philosophy

By

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Under the supervision and direction of

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Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Summary

Rapeseed is one of the world's major oilseeds, and Europe is the largest producer. During the extraction of the oil, a low economic-value by-product, rapeseed meal, is produced. Rapeseed meal is ordinarily used as an animal feed. It is rich in bioactive phenolics, including sinapinic acid, ferulic acid, caffeic and Ƥ-coumaric acid. Sinapinic acid has been shown to possess various bioactive properties including anti-diabetic, anti-inflammatory, anti-cancer and anti-oxidant activities. However, the amount of SA required to exert its beneficial effects exceeds what is obtainable from dietary intake alone. As such, the aim of this project was to isolate SA from a natural source, Irish rapeseed meal, to prepare extracts enriched with SA which could be consumed to prevent human disease.

To achieve this aim, a survey of the literature was performed in order to determine a suitable extraction methodology, given previous studies have focused on the extraction of total phenolics, rather than SA alone. The rapeseed meal was first processed by freeze-drying to remove excess moisture before being defatted with hexane. The method by Naczk *et al.* 1992, which involves the use of a solvent mixture of methanol: acetone: water (7:7:6 v: v: v) was used to prepare a phenolic extract containing SA. The total phenolic content of the prepared extract was estimated using the Folin-Ciocalteu assay, while its SA content was determined using ^{1}H-NMR spectroscopy. The yield of SA was determined to be 0.053 mg/mL

of SA (5.3% w: v). As the yield of this extract was low in terms of dry weight and yield of SA content, the initial extraction methodology was modified. The key modification was the direct hydrolysis of the meal itself, rather than hydrolysis of the solvent extract. Sonication and centrifugation steps were also added to remove excess oil from the extract. This reduced the dry weight of the extract and as such the yield of SA was increased to 0.569 mg/mL of extract (57% w: v), as estimated by mass spectrometry analysis.

The second aim of this work was to identify potential health benefits of the generated extracts, specifically heart health and anti-inflammatory activities using *in-vitro* and cell culture assays. The enzyme ACE-I (EC 3.4.15.1) plays a key role in hypertension and heart health. ACE-I is a zinc-dependent peptidase which cleaves angiotensin I (a vasodilatory peptide) to angiotensin II, which is a vasoconstrictor peptide involved in regulating blood pressure. A simple plasma ACE-I inhibition assay found that both extracts possessed ACE-I inhibitory activity, as did commercial SA.

The toxicity of the extracts and commercial SA were assessed using physiologically relevant cell lines. Furthermore, the anti-diabetic potential of the extracts were also assessed. GLUT4 is a key regulator of insulin-dependent glucose uptake, and its dysfunction can lead to the development of type 2 diabetes. As GLUT4 has been shown to be epigenetically regulated, histone deacetylase inhibitor (HDACi) assays and Western Blot analysis was performed to

determine the HDACi activity of SA. Our results found that commercial SA does not act as a HDACi. Polymerase chain reaction (PCR) and qPCR analysis revealed no effects on GLUT4 mRNA expression after both 24 and 48 hour treatments. A flow cytometry based fluorescent glucose uptake assay revealed the extracts inhibited glucose uptake, while commercial SA increased uptake in the H9c2 muscle cell line.

Inflammation plays a key role in the pathology of various diseases including diabetes, cancer, arthritis and heart disease. Preventing or reducing chronic systemic inflammation could therefore provide symptomatic relief or prevent these ailments. Primary human peripheral blood mononuclear cells (PBMCs) were used as a cellular model which is more representative of the *in-vivo* setting. PBMCs were isolated from buffy coats, and treated with the extracts and commercial SA, followed by the addition of LPS. ELISA assays showed that both extracts and commercial SA significantly reduced the expression of the pro-inflammatory cytokines TNF-α, IL-12, IL-6 and IL-1β.

This project resulted in the:

1. Successful generation of extracts containing SA from Irish rapeseed meal using an efficient extraction procedure
2. Extracts which possess bioactivities relevant to human health
3. This approach may be suitable for valorisation of rapeseed meal for health benefits

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Abbreviations

ANOVA	Analysis of variance
ACE-1	Angiotensin-converting enzyme I
AOAC	Association of Official Analytical Chemists
MDA	Cardiac malondialdehyde
CVD	Cardiovascular disease
CAGR	Compound annual growth rate
CK-MB	Creatine kinase
CTCL	Cutaneous T cell lymphoma
COX	Cyclo-oxygenase
DMSO-d6	Dimethylsulfoxide-d6
DMSO	Dimethylsulfoxide
ESI	Electrospray ionisation
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylene-diamine tetra-acetic acid
FAE	Ferulic acid esterase
F-C	Folin-ciocalteu
GLUT4	Glucose transporter type-4
HPLC	High-performance liquid chromatography
HDACi	Histone deacetlyase inhibitor

HDAC	Histone deacetylase
HIV-1	Human immunodeficiency virus type-1
HCL	Hydrochloric acid
IMS	Industrial methylated spirits
IBD	Inflammatory bowel disease
IκBα	Inhibitory kappa B
I/R	Ischemia/reperfusion
ISO	Isoproterenol
ITC	Isothiocyanates
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LDL	Low density lipoprotein
MS	Mass spectrometry
m/z	Mass/charge
MI	Myocardial infarction
NEFAs	Non-esterified fatty acids
NSCLC	Non-small cell lung cancer
NSAID	Non-steroidal anti-inflammatory drug
NMR	Nuclear magnetic resonance
Nf-κB	Nuclear transcription factor kappa-B

OA	Osteoarthritis
PRR	Pattern-recognition receptor
P/S	Penicillin/streptomycin
PBMCs	Peripheral blood mononuclear cells
PI-PRE	Phellinus igniarius
PMA	Phorbol 12-myristate-13-acetate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCE	Polyphenol clove extract
qPCR	Quantitative polymerase chain reaction
GIR	Rate of glucose infusion
Rcf	Relative centrifugal force
RP	Reversed-phase
RT-PCR	Reverse-transcription polymerase chain reaction
RA	Rheumatoid arthritis
RT	Room temperature
RPMI	Roswell Park Memorial Institute
SIRT 1	Silent information regulator 1
SA	Sinapinic acid
SAHA	Suberanilohydroxamic acid

NaOH	Sodium hydroxide
STZ	Streptozotocin
TMS	Tetramethylsilane
TLC	Thin layer chromatography
TLR	Toll-like receptor
TPC	Total phenolic content
TSA	Trichostatin A
TNBS	Trinitrobenzenesulfonic acid
TTC	Triphenyl tetrazolium chloride
TEB	Triton extraction buffer
SEM	Standard error of the mean
T2DM	Type 2 diabetes mellitus
UV/VIS	Ultraviolet/visible
WHO	World health organisation

Units

%	percent
°C	degrees celsius
bp	base pairs
cm	centimetre
g	gram
L	litre
mA	milliamp
M	molar
mg	milligram
mg/kg.b.w	milligram per kilogram of body weight
min	minute
ml	millilitre
mM	millimolar
nM	nanomolar
p	probability
RPM	revolutions per minute
µg	microgram
µl	microliter
µM	micromolar
V	volts
v	volume
v/v	volume per volume

w/v	weight per volume
X	magnification

Communications

Publications

Quinn, L, Gray, S.G, Meaney, S., Finn, S., Kenny, O., and Hayes, M (2017). Sinapinic and protocatechuic acids found in rapeseed: isolation, characterisation and potential benefits for human health as functional food ingredients. Irish Journal of Agricultural and Food Research, 56 (1), 104-119.

Quinn, L., Gray, S.G, Meaney, S., Finn, S., McLoughlin, P., and Hayes, M (2017). Extraction and Quantification of Sinapinic Acid from Irish Rapeseed Meal and Assessment of Angiotensin-I Converting Enzyme (ACE-I) Inhibitory Activity. Journal of Agriculture and Food Chemistry, 65 (32), pp 6886–6892

Poster presentations

Quinn, L., Gray, S.G, Meaney, S., Finn, S., and Hayes, M. Extraction of sinapinic acid from oilseed rape and assessment of its potential as a functional food ingredient for improved health benefit in the Irish populace. The International Union of Food Science and Technology (IUFoST), 2016. Ireland.

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meal and its effect on GLUT4 expression. 46th Annual Food Research Conference, 2017, Ireland.

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General Introduction

This chapter forms part of the published work:

'Sinapinic and protocatechuic acids found in Rapeseed: Isolation, characterisation and potential benefits for human health as functional food ingredients', Quinn, L *et al*. Irish Journal of Agricultural and Food Research, **2017**, 56 (1).

1.1 Introduction

The concept that constituents in our diet can promote health benefits has gained interest in recent years. This is evidenced by the development of functional foods, which is currently a widely examined area of food research. Functional foods can be defined as 'Natural or processed foods that contains known or unknown biologically-active compounds, which, in defined, effective non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease'(Martirosyan and Singh, 2015).

Rapeseed is one of the world's major oilseeds, with Europe being the largest producer with 20 million metric tonnes per year. The process of pressing the seeds to produce rapeseed oil results in a low-economic value by-product, rapeseed meal. Rapeseed meal is commonly used as animal feed, however it is rich in bioactive phenolic compounds, including sinapinic acid. Isolation of bioactive phenolics from a by-product of rapeseed oil production is largely in agreement with the current concept of the circular economy and total utilisation of crop harvest using a biorefinery approach.

Sinapinic acid has been shown to possess various bioactive properties including anti-diabetic, anti-inflammatory and histone deacetylase inhibitory activities (Senawong *et al.*, 2013; Kanchana *et al.*, 2011; Yun *et al.*, 2008). However, the amount obtained from our diet is insufficient to produce beneficial effects on health.

Therefore, isolating these bioactive phenolics from a natural source, such as rapeseed meal, could generate extracts containing concentrated amounts of phenolic acids which could be consumed as functional food ingredients to prevent health-related disease.

This chapter collates information concerning traditional methods to isolate and characterise phenolic compounds, with the focus on sinapinic acid (SA), from rapeseed meal. The potential utility of SA as a functional food ingredient is also mentioned, with the various bioactive properties of SA pertaining to human health and disease also discussed.

1.2 Rapeseed meal

Rapeseed (*Brassica napus*) is one of the world's major oil seeds, and one of the largest sources of vegetable oil, second only to soy and palm oils. In Europe, rapeseed oil (also known as Canola oil) is mainly utilised in the production of biodiesel, while in countries such as Canada and India it is used as a food source (Lomascolo *et al.*, 2012). The production of rapeseed oil involves mechanical extraction by pre-pressing and extrusion of rapeseeds, followed by extraction with solvents such as hexane (Booth, 2004). The processing of oil and meal involves various steps including seed preconditioning and flaking, seed cooking, pressing, solvent extraction along with meal desolventizing and toasting. Rapeseed oil can also be extracted from the seeds through cold-pressing, which

excludes the hexane extraction step (Canada, 2014). After the solvent extraction step the product is known as crude oil. Rapeseed meal is a by-product of this de-oiling process, and most commonly used as animal feed. Rapeseed meal has excellent nutritive values; it contains 40% proteins, while being high in fibre and rich in minerals such as calcium, magnesium, zinc and copper. It also contains a number of vitamins, including several B vitamins and choline, along with bioactive compounds such as tocopherols (vitamin E) (Vuorela, 2005). However rapeseed meal also contains antinutritional factors including phenolics, glucosinolates and phytates which present a major hurdle in terms of its use as a human food source. These antinutritional constituents lead to undesirable properties, such as inferior physiochemical properties, poor digestibility, unpleasant colour and poor taste (Tan *et al.*, 2011).

The major antinutritive components in rapeseeds are phenolic acid esters (e.g. sinapate esters), and their concentration in rapeseed is reported to be 30 times higher than that of soybean (Tan *et al.*, 2011). Sinapate esters lead to a dark colour and bitter taste in rapeseed meal, while complexes with proteins through oxidation during the processing of the oil decrease the digestibility of rapeseed meal. The amount of sinapate ester found in rapeseed meal is dependent on genetic variation; however it is estimated to be in the range of 0.005-0.0177 µg/g seeds of *Brassica napus* (Tan *et al.*, 2011). Rapeseed is reported to contain more phenolic compounds than any other oilseed plant. After the extraction of rapeseed oil, the majority of the

phenolic compounds remain in the meal. These phenolic compounds can be categorized into various sub-groups: phenolic acids, lignans, stilbenes, flavonoids, isoflavonoids and complex phenolic polymers (Vuorela, 2005). Phenolic acids tend to be found in a soluble form, conjugated with sugars or organic acids, and are usually constituents of more complex structures like lignans and hydrolysable tannins (Cheynier *et al.*, 2013).

1.3 Phenolic compounds in rapeseed

In rapeseed, the main phenolic compounds are derivatives of hydroxycinnamic acids (Vuorela, 2005; Cvjetko *et al.*, 2009). The biosynthetic origin of both benzoic and cinnamic acid derivatives is the aromatic amino acid L-phenylalanine, which itself is the end product of the Shikimate pathway (Robbins, 2003). The preceding conversion of L-phenylalanine to various hydroxycinnamic acids involves a 3-step sequence, referred to as the 'general phenylpropanoid metabolism' (Robbins, 2003). Phenolic compounds found in rapeseed mainly exist in esterified form. Sinapine, which is the choline ester of SA, is the main phenolic ester in rapeseed. However SA can also be esterified to other phenolic acids, sugars or kaempferols. The composition of esterified phenolic acids is influenced by cultivation and growing conditions as well as genetics (Vuorela, 2005). Frequently cultivated rapeseeds, including

canola (*Brassica napus* and *Brassica campestris*) have similar amounts of phenolic compounds. Phenolics are mainly located in the seed cotyledons, with trace amounts present in the seed coats (Kozlowska *et al.*, 1990). Depending on the growing conditions and degree of maturation, rapeseed can contain approximately 0.39-1.06% sinapine. Sinapine plays an important role in rapeseed, acting as storage for both SA and choline in young plants (Kozlowska *et al.*, 1990). As the seeds mature, some of the sinapine is enzymatically hydrolysed by sinapine esterase to choline and free SA (Tzagoloff, 1963). SA is a preliminary compound in the synthesis of lignins and flavonoids, while choline is a key metabolic product of the methylation cycle (Vuorela, 2005). Apart from being esterified, free phenolic acids can also be found in rapeseed, comprising up to 15% of the total phenolics in rapeseed meal. SA constitutes 70.2-85.4% of free phenolic acids in defatted rapeseed meals, however other phenolics acids can also be found such as ferulic acid, p-coumaric, caffeic and protocatechuic acid (Vuorela, 2005).

1.3.1 Sinapinic acid (SA)

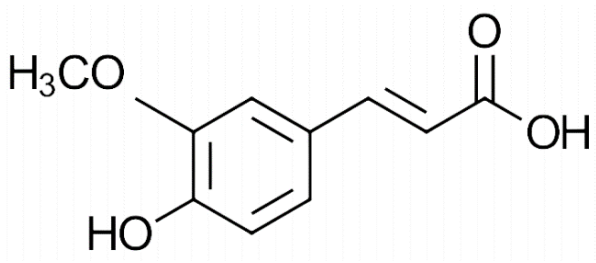
Sinapinic acid, (- or sinapic acid (SA)) as it is also known, is present throughout the plant kingdom. Citrus fruits contain differing levels of SA, with lemon and Murcott orange known to contain the highest

quantities of SA (Nićiforović and Abramovič, 2014). Cereal grains like rye and rice also contain substantial amounts of SA, while it is also present in various herbs such as sage and thyme. SA and its derivatives are particularly abundant among the different varieties of *Brassica* vegetables, of which rapeseed is a member, such as kale, white cabbage, turnip and broccoli (Nićiforović and Abramovič, 2014). As such, SA is a common component of the human diet.

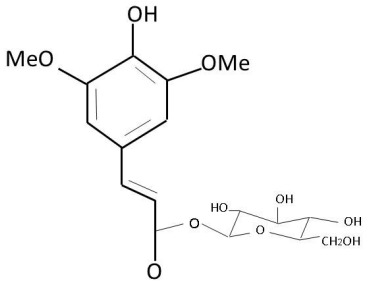
In terms of its structure, SA is a 3, 5-dimethoxy-4-hydroxycinnamic acid (Figure 1), which is found either in free or esterified form. 1-O-β-D-glucopyranosyl sinapate is the most common glycoside of SA, and can be found in numerous Brassicaceae species (Nićiforović and Abramovič, 2014). SA can also form dimers with itself and with ferulic acid in the walls of cereal cells, while more recently 8-8-coupled sinapinic acid dehydrodimers and 3 sinapate-ferulate heterodimers have been discovered in various insoluble and soluble cereal grain dietary fibres (Nićiforović and Abramovič, 2014). SA in rapeseed also exists as the glucosidic ester, glucopyranosyl sinapate. SA does not usually exist in free form, and less than 16% of SA is present as free SA (Vuorela, 2005). Rapeseed contains 240-590 μg/g of SA, while rapeseed cake contains approximately 170-454 μg/g of SA (Niciforovic *et al*. 2014). 4-Vinylsyringol is a decarboxylation product of SA which is found in crude rapeseed oil. During rapeseed oil extraction, elevated temperatures and pressure causes SA to undergo structural changes, resulting in the formation of 4-vinylsyringol, a decarboxylation product of SA, along with

syringaldehyde (Nićiforović and Abramovič, 2014). The amount of SA derivatives (which include: sinapoyl glucose, methyl sinapate, sinapoyl malate and 1-sinapoyl-2-feruloylgentiobiose) found in rapeseed ranges between 6390 and 18370 µg/g, but this is dependent on the method used to process the oil (Nićiforović and Abramovič, 2014; Lomascolo *et al.*, 2012).

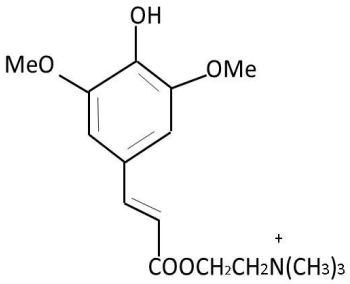
Until recently, other hydroxycinnamic acids such as ferulic and caffeic acid have been the focus of research due to their demonstrated antioxidant properties and reported health benefits (King *et al.*, 1999; Koshihara *et al.*, 1984). The caffeic acid derivatives dicaffeoylquinic and dicaffeoyltartaric acid have been shown to be potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) integrase and to inhibit HIV replication at non-toxic doses in laboratory conditions (King *et al.*, 1999). Caffeic acid has also been shown to inhibit the biosynthesis of leukotrienes, which are immunoregulatory mediators involved in diseases such as asthma, inflammation and allergic reactions (Koshihara *et al.*, 1984). However evidence is accumulating that SA could have potential significant health effects.



Sinapinic acid



Sinapate (glucopyranosyl sinapate)



Sinapine

Figure 1. Chemical structures of sinapinic acid and its derivatives sinapate and sinapine.

1.4 Methods of phenolic acid extraction from rapeseed meal

1.4.1 Sample preparation

When extracting plant phenolics such as SA, samples including rapeseed meal or seed are typically air-dried or freeze-dried. Standard freeze-drying is the preferred method due to its superior preservation of the phenolic content in the samples, but it is regarded as expensive (Dai and Mumper, 2010). Following freeze-drying, samples are then milled, ground and homogenized. However before

phenolic extraction can take place, rapeseed samples must be defatted, due to their high oil content (approximately 40%). The most common method of defatting is extraction with hexane using a Soxhlet apparatus (Vuorela, 2005; Klockeman, Toledo and Sims, 1997). Residual oil can be removed by using Soxhlet extraction for 5 to 6 hours with petroleum ether as the solvent (Rodrigues, Carvalho and Rocha, 2014). However, rapeseed cake was also defatted using alcohol: benzene (1:1 v: v) in a Soxhlet apparatus for 6h at 80°C (Jeong *et al.*, 2013). Methanol/ammonium/water or carbon tetrachloride have also been utilised for defatting (Vuorela, 2005).

1.4.2 Solvent extraction of SA

Solvent extractions are the most common method used to isolate phenolics such as SA from rapeseed oil and meal. Solvents such as methanol, ethanol and acetone are often used. Success in the retrieval of phenolic compounds is influenced by two key parameters, the extraction time and temperature (Dai and Mumper, 2010). Optimisation of these parameters is essential in order to ensure the highest yield and best quality product is obtained. If the extraction temperature is increased, this can promote higher analyte solubility by increasing the solubility and mass transfer rate (Dai and Mumper, 2010). Furthermore, at higher temperatures, both the viscosity and surface tension of the solvents are decreased, helping the solvent to reach the sample and thus increasing the extraction rate. However it must be noted that numerous phenolic compounds are easily

oxidized and hydrolysed. High temperatures and long extraction times can also increase the oxidation of phenolic compounds, and decrease the phenolic yield in the extract (Dai and Mumper, 2010). A recent study used 70% methanol, 70% isopropanol or 70% ethanol along with ultra-sonication in order to extract phenolics from canola seed, meal and press cakes (Khattab *et al.*, 2010). It was reported that extraction with 70% methanol at room temperature was the most efficient of the conditions tested; producing the highest yields of both total phenolics as well as the major phenolics like sinapine and SA. Defatted canola seeds were found to contain 90- 590 µg of SA, the cakes contained 290-440 µg and the meal contained 320-410 µg of SA (Khattab *et al.*, 2010). Liu *et al.* (2012) detected SA in Chinese rapeseed hulls and dehulled flour, extracted the phenolic compounds using 80% methanol at 25°C, with similar efficacy as extraction with 70% methanol, yielding an average 158 µg of SA (Liu *et al.*, 2012; Qiu, Liu and Beta, 2010). In contrast, another study varied their approach, using four different conditions when extracting phenolic acids from canola seed and meal (Cai and Arntfield, 2001). They found the most effective methods for extracting sinapine and SA were extraction using 70% methanol at 75°C for 20 minutes and refluxing with 100% methanol for 20 minutes. Both of these extraction conditions were found to be superior to the other two conditions used (100% methanol at both 50 and 75°C).

1.4.3 Hydrolysis of SA

In rapeseed, SA mainly exists in esterified form, therefore hydrolysis is necessary to release the bound and esterified phenolics once they have been extracted (Krygier, Sosulski and Hogge, 1982; Vuorela, 2005). An example of this is the hydrolysis of sinapine to SA. Alkaline-acid hydrolysis can be carried out by first using 2 or 4 M sodium hydroxide (NaOH), and subsequently lowering the pH below 2 using hydrochloric acid (HCL) (Khoddami, Wilkes and Roberts, 2013; Liu *et al.*, 2012). At this pH, the released phenolics exist as phenolic acids and can subsequently be extracted with diethyl ether or diethyl ether/ethyl acetate (1:1). In order to analyse SA and its derivatives, hydrolysis of sinapine to SA is the favoured method, as sinapine is not available as a commercial standard (Vuorela, 2005).

Recently, there has been a shift toward enzymatic hydrolysis of esterified phenolics, using enzymes such as Ultraflo L and ferulic acid esterase (FAE) (Vuorela, Meyer and Heinonen, 2003). One study carried out enzymatic hydrolysis using Ultraflo L, FAE, Viscozyme L and Cellulast (Vuorela, Meyer and Heinonen, 2003). Hydrolysis was carried out under nitrogen in a shaking water bath at 37°C, except for Cellulast which was hydrolysed at 50°C. The group found that Ultraflo L and FAE were the most efficient enzymes in terms of their yields of both total phenolics and SA (Vuorela, Meyer and Heinonen, 2003).

1.5 Analysis and characterisation of rapeseed phenolics

1.5.1 Spectrophotometric assays

Many of the methods available to determine total phenolic content in plant samples are spectrophotometric assays, whereby the phenolic compounds react with a colorimetric reagent which can be measured in the visible portion of the spectrum (Ainsworth and Gillespie, 2007). The Folin-Ciocalteu (F-C) assay is based on the transfer of electrons in an alkaline medium from the phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, thus forming blue complexes which can be measured spectrophotometrically at 760 nm (Ainsworth and Gillespie, 2007). The exact chemistry of the reaction is unknown, but it is thought to contain heteropolyphosphotunstates-molybdates. Sequences of reversible one-or two-electron reduction reactions lead to the formation of blue species, possibly $(PMoW_{11}O_{40})^{4-}$ (Huang, Ou and Prior, 2005). One drawback with this method is that the F-C reagent is non-specific for phenolic compounds, and can be reduced by various non-phenolic compounds. Ascorbic acid easily reacts with the F-C reagent, and it is therefore recommended that its content is measured before the addition of the alkali or by using a more specific assay, and then subtracted from the F-C value obtained (Ainsworth and Gillespie, 2007). As phenolic compounds will only react with the F-C reagent under basic conditions, the solution must be adjusted to a pH of approximately 10 using sodium carbonate (Huang, Ou and Prior, 2005). The F-C reagent must be added before the alkali, as

oxidants or air oxidation after the sample has been made alkaline could inhibit the F-C reagent (Ainsworth and Gillespie, 2007). Overall, this is a relatively simple, fast and economical method for determining total phenolic contents in numerous samples.

1.5.2 High performance liquid chromatography (HPLC)

Chromatography exploits subtle differences in certain physical properties of the molecules, such as their solubility in water, their charge and size (Bird, 1989). HPLC is the preferred method to separate and quantify phenolic compounds from rapeseed extracts, for two main reasons; there is a wide range of commercially available columns, - and the potential to combine two or more columns in a 'switching mode' (Stalikas, 2007). The principle of column chromatography is that molecules interact to and thus partition to different degrees with the mobile phase (i.e. the solvent) and the stationary phases (i.e. the column) (Bird, 1989). Molecules which interact strongly with the column will be retained and so will move more slowly than molecules which interact more strongly with the solvent, and thus elute later on (Bird, 1989). The introduction of reversed-phase (RP) columns has significantly improved HPLC separation of various classes of phenolic compounds. More or less exclusively, reverse-phase C_{18} columns range from 100 – 250 mm in length, with an internal diameter of 3.9 – 4.6 mm (Stalikas, 2007). Typically, HPLC analysis of phenolic compounds is performed with

an ambient column temperature, but temperatures slightly higher in the range of 30 – 40^0C have also been recommended (Stalikas, 2007). In terms of the mobile phase utilised, isocratic (i.e. the composition of the solvent remains unchanged) and gradient elution (the solvent strength is gradually increased) are applied when analysing phenolic compounds. The most common organic modifiers are acetonitrile and methanol, however methanol is typically preferred. In chromatography, analytes of like nature interact the best with one another. RP columns typically comprise of non-polar material, meaning nonpolar analytes will have enhanced retention. Analytes which are ion- suppressed are deemed to be more nonpolar than polar, meaning they will have superior retention on the nonpolar stationary phase of HPLC apparatus. In order to maintain an analyte in an ion-suppressed state, the pH of the mobile phase must be at least two units below its pK_a value. The majority of phenolic acids have a pK_a of about 4, and as such, the recommended pH range for the HPLC assay is 2-4 (Stalikas, 2007). By maintaining the acidic phenolic analytes in an acidic environment, the analyte can retain its protons, and thus remains ion- suppressed. This results in the improved resolution and reproducibility of retention characteristics.

Phenolics are commonly detected using photodiode array or ultraviolet/visible (UV/VIS) detectors, as they typically absorb in this region. Phenolic acids which contain the benzoic acid carbon framework absorb maximally in the 200 – 290 nm range, while those

based on cinnamate derivatives have a broader range from 270 – 360 nm (Stalikas, 2007). For dual monitoring when separating mixtures of phenolic acids, 254 and 280 nm, or 280 and 320nm are typically used.

Cai and Arntfield (2001) separated phenolics from canola seeds and meal using a reverse-phase C_{18} column, using an acetate buffer or methanol as the mobile phase (Cai and Arntfield, 2001). Two gradients were used, the first was a 10 minute isocratic-linear-concave gradient, and SA was eluted during the isocratic flow. The second was a 15 minute isocratic-linear gradient, and once again SA was eluted during the isocratic flow and identified using HPLC (Cai and Arntfield, 2001).

1.5.3 Electrospray Ionization Mass spectrometry

Mass spectrometry is an analytical technique which is capable of performing both qualitative and quantitative analysis of 'analyte molecules', i.e. both its structure and molecular mass/concentration, respectively (Ho *et al.*, 2003). The analyte molecules of interest must first be ionized, where they acquire either a positive or negative charge. The ions are then able to pass through the mass spectrometer, where they arrive at various parts of the detector according to their mass/charge (m/z) ratio (Ho *et al.*, 2003). The results are then displayed as a mass spectrum which displays the relative abundance of the signals according to their m/z ratio. The most commonly used ionization technique is electrospray ionization, and this is the

preferred ionization source when analysing liquid samples. Electrospray ionization is compatible with traditional chromatographic separation techniques, and given the ions generated are stable rather than in an excited state, this prevents their rapid decay (Wilm, 2011). ESI-MS has also been shown to play an important role in the screening of phenolics and flavonoids (Saldanha, Vilegas and Dokkedal, 2013). Different phenolic acids, including sinapinic acid, were identified in commercial rapeseed oils and deodistillate, a by-product of the refining process, using ESI-MS (Harbaum-Piayda *et al.*, 2010). Mass spectrometry was also used in order to detect, characterise and identify phenolic acids in extracts obtained from Danshen, which is the dried root of *Salvia miltiorrhzia* used in Chinese medicine (Liu *et al.*, 2007).

1.5.4 Nuclear Magnetic Resonance (NMR) spectroscopy

Although not originally used for this purpose, NMR spectroscopy was found to be a useful technique in order to determine the molecular structure of organic compounds (Bruice, 2006). High resolution ^{1} NMR has recently gained attention as a useful method for analysing complex mixtures without prior separation of its components. This method of analysis is non-invasive and quick, with only one measurement needed. There is also no need to pre-treat the sample before running the analysis and the integrity of the sample is maintained (Christophoridou and Dais, 2008). Nuclear

Magnetic Resonance spectroscopy is based on the principle that many nuclei have spin, and all are electrically charged. When an external magnetic field is applied, energy transfer is possible between a low energy 'alpha-spin state' and higher energy 'beta-spin state' (Bruice, 2004). The energy emitted during this transfer is used to generate an NMR signal, which forms part of the NMR spectrum. The resulting NMR spectrum plots the intensity of the NMR signals vs the magnetic field with reference to Tetramethylsilane (TMS), a reference compound (Bruice, 2004).

Various studies have used ^{1}H-NMR in order to identify and quantify phenolic acids. A study by Christophoridou and Dais (2008) used ^{1}H-NMR to identify constituents in the phenolic fraction derived from olive oil. A recent study also examined the phenolic constituents in a product derived from the rapeseed oil refining process. NMR revealed that SA, along with its derivatives, were found to be the predominant phenolic acids present (Li and Guo, 2016). NMR also proved useful in determining the interactions between phenolic acids in mango and papaya (López-Martínez *et al.*, 2015).

1.6 Potential benefit of Sinapinic Acid as a functional food ingredient

Now, more than ever, people are aware of what they eat and the associated health benefits that come from consuming a healthy and

balanced diet. Functional foods are classified as foods which are enriched, fortified or enhanced and provide health benefits beyond essential nutrients, when consumed at efficacious levels on a regular basis (Hasler, 2002; Vo and Kim, 2013). A phenolic acid found in rapeseed, sinapinic acid (SA), has the potential to improve human health through diet, specifically as a functional food ingredient.

Epidemiological and case-control studies suggest that consumption of *Brassica sp* (cruciferous) vegetables reduces the risk of various cancers, including lung, breast, liver, colon, prostate and pancreatic, as well as cardiovascular disease and cerebrovascular disease including ischaemic stroke and intracerebral haemorrhage (Sahu and Srivastava, 2011; Zhang *et al.*, 2011; Mizrahi *et al.*, 2009). These beneficial effects are believed to be mediated by bioactive molecules, including phenolic compounds and isothiocyanates (ITCs), released upon consumption of the containing foodstuffs. SA is present in Chinese compound herbal medicines, including 'Yinhuang powder', which is known for its anti-inflammatory activities and 'Chenxiang-Shuqi pill', a prescription which is used to treat dyspepsia, abdominal pain and hypochlorhydria (Wen *et al.*, 2005).

However as previously discussed, SA in our diet is present in insufficient quantities in order to exert a sufficient physiological effect. Valorising rapeseed by-products to extract SA requires stabilization of the phenolic compounds before application in either

food or cosmetics. Indeed, a patent regarding SA for cosmetic use, including as a topical composition to reduce the signs of aging, noted the instability of SA, and proposed microencapsulation as a solution (Markert *et al.*, 2011).

There has been success in overcoming this issue, involving another phenolic acid, protocatechuic acid. An *in-vitro* study demonstrated the potential to successfully encapsulate PCA using chitosan nanoparticles for functional food purposes (Madureira, Pereira and Pintado, 2016).

1.7 Bioactive properties of sinapinic acid

1.7.1 Histone deacetylase inhibitory activity

Epigenetics refers to heritable changes in gene expression that do not involve changes in the underlying DNA sequence (Brunet and Berger, 2014). These changes include post-translational histone modifications, including histone methylation and acetylation. Histone deacetylase enzymes (HDACs) remove acetyl groups from histones, resulting in a 'closed' chromatin conformation which blocks gene transcription and therefore gene expression (Tortorella *et al.*, 2015). Aberrant HDAC activity is common in various cancer types, driving carcinogenesis through the repression of genes involved in the cell cycle, cellular differentiation and apoptosis. Vital tumour suppressor genes, such as p21, are also targets for HDAC-mediated transcriptional silencing (Tortorella *et al.*, 2015).

There has been some success in developing clinical therapeutics using HDAC inhibition, for example the HDAC inhibitor SAHA/vorinostat was approved for the treatment of cutaneous T cell lymphoma (CTCL) in 2006. A natural product, romidepsin, was approved to treat CTCL in 2009 (Mair, Kubicek and Nijman, 2014). As such, HDACi are emerging as potential therapeutic targets. Recently, Senawong *et al*. (2013) extracted phenolic compounds from the rhizome of *H. formicarum Jack*. The phenolic extract obtained was analysed by RP-HPLC, and SA was found to be one of its major components (Senawong *et al.*, 2013). SA was found to block HDAC enzyme activity *in vitro* by a greater amount than that of a well-established HDAC inhibitor sodium butyrate, with IC_{50} values of 2.27 mM for SA and 0.97 mM for sodium butyrate (Senawong *et al.*, 2013). The authors suggest that SA accounts, at least partly, for the inhibition of HDAC activity by the *H. formicarum Jack* phenolic extract. The phenolic-extract possessing HDAC inhibitory activity was found to inhibit the growth of HeLa cells in a dose- and time-dependent manner, while SA was also found to significantly inhibit the growth of HeLa cells. The phenolic extract and SA were also found to have a significant effect on the induction of apoptosis, up to 78.9% and 8.4%, respectively (Senawong *et al.*, 2013). The results obtained suggest that the phenolic extract and SA suppress the growth of HeLa cells through the induction of apoptosis (Senawong *et al.*, 2013).

1.7.2 Protective role in cardiovascular disease

Cardiovascular disease (CVD) is the number one cause of death globally, with the World Health Organisation (WHO) estimating 17.5 million deaths in 2012 alone, a figure which represents nearly one third of all global deaths. However, many cardiovascular diseases may be prevented with lifestyle modifications, such as healthy diet, weight loss, exercise and no tobacco use (Organisation, 2019).

A major precursor for the development of atherosclerosis, which is the hardening of the arteries, is the oxidation of low density lipoprotein (LDL) (Poloni, Dangles and Vinson, 2019). A multicentre study was carried out titled 'Primary Prevention of Cardiovascular Disease with a Mediterranean Diet' PREDIMED. The results have shown that the risk of CVD, along with diabetes, were significantly reduced when a diet containing polyphenols was consumed (Poloni, Dangles and Vinson, 2019). A recent study by Poloni, Dangles and Vinson (2019) investigated the binding of polyphenols, including phenolic acids such as sinapinic and protocatechuic acid, to LDL through fluorescence quenching methods. The results revealed that polyphenols and their metabolites bind more effectively to albumin than LDL. However, it has also been shown that the small proportion of polyphenols which do bind to LDL are 'cardioprotective' (Poloni, Dangles and Vinson, 2019). The group hypothesize that many of the health benefits attained from consuming a diet rich in polyphenols is a result of their binding to

proteins including albumin and lipoproteins, as well as binding to receptors involved in processes such as inflammation (Poloni, Dangles and Vinson, 2019).

Myocardial infarction (MI) induction with isoproterenol (ISO) is a well-established animal model used to evaluate the effects of various cardioprotective agents. This model is also excellent as the pathophysiological changes are analogous to those taking place in humans with MI. ISO is a synthetic catecholamine, which in large doses results in severe stress in the myocardium, leading to necrosis of the heart muscle (Roy and Prince, 2012). The auto-oxidation of catecholamines generates cytotoxic free radicals, and this, coupled with the physiological imbalance between the production of free radicals and antioxidant defence systems, are considered critical factors in the loss of integrity and function of myocardial membranes during ISO-induced MI (Roy and Prince, 2012). Lysosomal enzymes are also known to play an important role in the inflammatory process, and the increased activity of these enzymes in ISO induced MI is believed to be responsible for tissue damage and infarcted heart.

In a recent study, the protective effects of SA were evaluated on isoproterenol- induced MI rats (Roy and Prince, 2012). Rats were orally treated with SA dissolved in 0.5 % DMSO at 12 mg/kg body weight by intragastric tube. Elevated serum creatine kinase (CK-MB) activity was found in the experimental animals. Pre- or co-

effect against ischemia reperfusion injury through activating silent information regulator 1 (SIRT1), a histone deacetylase, thereby decreasing myocardial infarct size, down-regulating the pro-apoptotic protein Bax and up-regulating the anti-apoptotic protein Bcl2 (Yang *et al.*, 2013). To determine the effects of SA on I/R injury, SA was dissolved in corn oil and orally administered to rats using an intragastric tube at 20 and 40 mg/kg body weight for 7 days. Oral pre-treatment with SA significantly improved percent rate pressure product and percent coronary flow when compared to untreated I/R hearts. Mitochondrial dysfunction results in cardiac contractile dysfunction and hypertrophy and under conditions of oxidative stress, the enzymes of the TCA cycle are disrupted. Decreases in the activity of several TCA enzymes were blunted after pre-treatment with SA. SA was also found to reduce H9c2 cardiomyoblast cell injury caused by H_2O_2, likely due to its scavenging abilities (Silambarasan *et al.*, 2015). Although the above studies are preliminary investigations, SA has shown itself to be a promising therapeutic option to treat cardiovascular diseases, warranted of further investigations.

1.7.3 Anti-diabetes activity

Diabetes Mellitus is a group of 'heterogenous, hormonal and metabolic disorders', with hyperglycemia being one of its physiological characteristics (Kanchana *et al.*, 2011). Type 2

diabetes mellitus (T2DM) is one of the most prevalent 'metabolic pathologies' worldwide, with more than 220 million people affected (Scazzocchio *et al.*, 2015). Of this 'diabetic population', over 90% suffer from T2DM (Nithya, 2017). The clinical management of DM involves the use of drugs including biguanides, sulphonylureas, thiazolidiones and insulin, either alone or in combination (Nithya, 2017). However, these drugs elicit adverse side effects such as upset stomach, anaemia and weight gain, with resistance commonly occurring after extended use. Therefore, identifying new drugs from natural sources with less side effects is a growing research topic of great importance.

Nithya (2017) studied the effects of SA on glucose homeostasis using high fat diet fed-low dose streptozotocin (STZ)-induced experimental T2DM in rats. SA was orally administered at 25 mg/kg.b.w for 30 days and the activities of carbohydrate metabolising enzymes in hepatic tissues were analysed. Treatment with SA was found to significantly revert the altered activities of the carbohydrate metabolising enzymes: glucokinase, pyruvate kinase, glucose-6-phosphatase, fructose-1, 6-biphosphatase, glucose-6-phosphate dehydrogenase and lactate dehydrogenase to near normal in the hepatic tissues of diabetic rats (Nithya, 2017). SA was also shown to significantly reduce the levels of fasting blood glucose and glycosylated hemoglobin, while the levels of both insulin and hemoglobin in the plasma were increased (Nithya, 2017). A key finding by the group was the efficacy shown by SA when compared

to Metformin, which is a biguanide drug used in the clinical treatment of diabetes. SA was administered at 25 mg/kg.b.w, yet its efficacy was comparable to Metformin, despite it being administered at a higher dose of 50 mg/kg.b.w.

As mentioned, hyperglycemia is one of the main physical characteristics of diabetes. Chronic hyperglycemia has detrimental cellular effects, as oxidation of glucose, protein glycation and glycoxidation all enhance the formation of ROS. In diabetes, protein glycation and glucose oxidation can generate free radicals which may cause lipid peroxidation (Kanchana *et al.*, 2011). A study on the biochemical and physiological effects of SA treatment on STZ-induced diabetic rats revealed that oral administration of SA was found to cause a significant improvement in their body weight, attributed to the anti-hyperglycemic effect of SA. SA also influenced other physiological parameters, such as decreasing kidney weight to near normal value (Kanchana *et al.*, 2011). Diabetic rats treated with SA had a significant decrease in plasma glucose when compared to control rats. The authors suggested this effect could be due to the regeneration of existing pancreatic β-cells by SA and enhanced transport of glucose to peripheral tissues. Treatment with SA significantly ($p < 0.05$) increased the levels of both C-peptide (4.34 ng/mL) and insulin (10.95 μU/mL) in STZ-induced diabetic rats compared to untreated controls (Kanchana *et al.*, 2011). In STZ-induced diabetic rats, treatment with SA was found to decrease the level of glycosylated hemoglobin and increase the level of total

hemoglobin. The authors speculate that these changes could be due to the reduction of blood glucose level (Kanchana *et al.*, 2011).

Increased activities of the carbohydrate metabolising enzymes glucose-6-phosphatase and fructose-1, 6-biphosphatase were observed in the liver and kidney of STZ-induced diabetic rats. However administration of SA restored their level of activity to near normal, resulting in improved glycemic control (Kanchana *et al.*, 2011).

SA has been found to significantly lower postprandial glucose, but did not alter plasma insulin levels, which suggests that SA has a direct influence on glucose utilization (Cherng *et al.*, 2013). GLUT4 (glucose transporter type 4) expression was significantly increased in the soleus muscle of the STZ rats (Cherng *et al.*, 2013). A radioactive tracer also revealed that SA caused a concentration-dependent increase in glucose uptake in the soleus muscle, and this was also confirmed in L6 myoblast cells, indicating that SA may directly stimulate glucose uptake. In order to study the effects of SA on insulin resistance, the group employed an animal model of type 2 diabetes by feeding rats a fructose chow-feed. Insulin resistance is indicated by the rate of glucose infusion (GIR), with a high GIR value indicative of insulin-sensitivity while a low GIR value indicates insulin resistance. Glucose infusion rate is a method of calculating the amount of carbohydrates received, which is expressed as milligrams of glucose per kilogram body weight per

minute. SA induced a higher GIR value in the fructose chow-fed rats, likely due to the enhanced glucose uptake in skeletal muscle (Cherng *et al.*, 2013).

A recent *in-vivo* study has also shown that SA can ameliorate the development of metabolic disorders induced by oestrogen deficiency in rats (Maria *et al.*, 2018). These results indicate that SA is a potential target for the treatment and management of DM.

1.7.4 Anti-inflammatory activity

An earlier study by Yun *et al*. (2008) characterised the effects of SA on LPS-induced pro-inflammatory molecules such as TNF-α, IL-1β, iNOS and PGE_2. The group found that in response to LPS, SA at 40, 80 and 160 μM reduced the expression levels of iNOS and COX-2 which were markedly upregulated, in a concentration-dependent manner in RAW 264.7 cells. Subsequent RT-PCR analysis demonstrated that the mRNA levels of both iNOS and COX-2 were also reduced in a dose-dependent manner, suggesting that they are also inhibited at a transcriptional level (Yun *et al.*, 2008). SA also reduced the production of TNF-α and IL-1β, and their levels of mRNA in a dose-dependent manner. The nuclear transcription factor kappa-B (NF-κB) plays a key role in the pathogenesis of inflammation, and as such there are numerous drugs designed to treat inflammatory disease which are based on the inhibition of NF-κB (e.g. non-steroidal anti-inflammatory drugs (NSAIDs) such as

Aspirin). The same study by Yun *et al*. (2008) found that RAW 264.7 cells treated with SA at 40, 80 and 160 μM had a reduction in the DNA binding activity of p65, preventing transcription initiation by NF-κB and subsequent expression of genes involved in the immune response (Schmitz and Baeuerle, 1991; Pahl, 1999). The nuclear translocation of both p65 and p50 was also inhibited. The above results demonstrate that SA inhibits NO, PGE_2, iNOS, TNF-α, COX-2 and IL-1β expression, and this is at least partly mediated via an NF-κB mechanism.

Further investigations also demonstrated that SA inhibited the LPS-induced degradation of IκB-α, thereby preventing the release of NF-κB *in-vivo*. Studies using serotonin and carrageenan-induced rat paw edema models showed that SA was able to inhibit edema caused by acute inflammation (Yun *et al.*, 2008).

The anti-inflammatory effects of SA were also shown *in-vivo* using trinitrobenzenesulfonic acid (TNBS)-induced colitis in Balb/c female mice. SA was found to decrease the levels of TNF-alpha and myeloperoxidase in the colonic tissue, while also significantly improving both colonic weight and length (Lee, 2018).

Li *et al*. (2019) have demonstrated that SA could play an important role in Osteoarthritis (OA) through its anti-inflammatory properties. Human chondrocytes which were pre-treated with SA had reduced levels of IL-1β-induced IL-6, prostaglandin E2, nitric oxide and TNF-alpha. As previously reported, SA inhibited NF-κB activation,

induced by IL-1β, through the nuclear factor-erythroid 2-related factor-2 (Nrf2)/heme oxygenase 1 pathway. *In-vivo*, the progress of OA in mice was delayed by treatment with SA (Li *et al.*, 2019).

1.8 Thesis aims

1. To generate and characterise extracts containing sinapinic acid from Irish rapeseed meal

2. To assess the potential anti-diabetic activities of commercial SA and SA-containing extracts

3. To evaluate the effects of commercial SA and SA-containing extracts on pro-inflammatory markers

Chapter 2

Materials and Methods

2.1 Chemicals and materials

All laboratory reagents and chemicals were stored according to the manufacturer's instructions. All reagents and chemicals were of analytical standard and purchased from Sigma Aldrich (Dublin, Ireland). These included: methanol, acetone, sodium hydroxide, sodium carbonate, resazurin sodium salt, Captopril®, Folin-Ciocalteu reagent, DMSO-d6 (with 1% TMS v/v), sinapinic acid, ammonium persulfate, and N, N. N', N'-tetramethylethylenediamine. All plastics were purchased from Thermo Fisher Scientific (Dublin, Ireland).

2.2 Rapeseed meal preparation

Rapeseed meal was kindly supplied by Donegal Rapeseed Company, Co. Donegal, Ireland. The meal was prepared from the seed harvested in the summer of 2014, which was dried and cold-pressed by Donegal Rapeseed Oil. The rest of the meal processing was performed at Teagasc Food Research Centre, Ashtown. The meal was placed into a freeze-drier (Cuddon Engineering, New Zealand) for 24 hours to remove any excess moisture. The freeze-dried rapeseed meal was then defatted overnight with hexane using a meal: solvent ratio of 90 g: 5 L (w/v) at room temperature on a JENWAY 1002 stirrer (Wolflabs, UK), and repeated three times. Defatted meal was subsequently air-dried in a chemical fume hood overnight.

2.3 Generation of sinapinic-containing extracts from defatted Irish rapeseed meal

2.3.1 Extraction method

Extraction was performed as described by Naczk *et al*. (1992), with modifications as described. Three fractions were generated (1-3) using this methodology. Phenolic acids were extracted from defatted rapeseed meal using a solvent mix of methanol: acetone: water (7:7:6 v: v: v), in a meal to solvent ratio of 2 g: 40 mL (w/v). The mixture was vortexed for 15 sec using a Vortex Genie (Scientific Industries, Inc.), and then centrifuged at 5000 rpm (3472 x g) for 15 min at room temperature (Sorvall LYNX 4000, Thermo Scientific, USA). A portion of this mixture was kept and evaporated to dryness using a rotary evaporator at 30°C (BUCHI, Switzerland); this was fraction 1. The remaining supernatant was kept to generate fractions 2 and 3. To generate fraction 2, a portion of the supernatant was extracted 6 times with diethyl ether: ethyl acetate (1:1 v: v), and evaporated to dryness as before. To generate fraction 3, the supernatant was hydrolysed with an equal volume of 4 M sodium hydroxide (NaOH) under nitrogen for 4 hours at room temperature. The hydrolysed solution was then acidified to below pH2 using 6 M hydrochloric acid (HCL) and extracted six times with an equal volume of diethyl ether: ethyl acetate (1:1 v: v). The fraction was then evaporated to dryness as before, and a labelled fraction 3 (also extract I).

2.3.2 Modified extraction method

A modified extraction method was developed, based on the initial extraction methodology. Phenolic acids were extracted by hydrolysing defatted rapeseed meal with 4 M NaOH using a meal to solvent ration of 100 g: 800 mL (w/v) for one hour at room temperature. The mixture was then centrifuged at 5000 rpm (3472 x g) for 10 min at room temperature, and the supernatant collected. The supernatant was then acidified to below pH2 using concentrated HCL. Ethyl acetate was then added at half the volume of the acidified solution, and mixed well. This solution was then centrifuged at 5000 rpm (3472 x g) for 5 min, and the supernatant collected. This was evaporated to dryness using rotary evaporation at 37 °C and labelled extract II.

2.4 Total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu (F-C) assay, according to the method described previously by Khattab *et al*. (2010). Briefly, the phenolic extracts (1-3) were prepared to a final concentration of 1 mg/mL with methanol, in triplicate (Sigma Aldrich, Ireland). Aliquots (0.2 mL) of each sample were diluted to 0.5 mL with water, and 0.5 mL of Folin-Ciocalteu phenol reagent (Sigma Aldrich, Ireland) was added. After 3 min., 1 mL of 19 % sodium carbonate was added to each sample. After 60 min., absorption was measured at 750 nm using a spectrophotometer (Hitachi U-2900). Sinapinic acid (Sigma Aldrich, Ireland) was used

as the standard (1 – 10 mg/mL) and results were expressed as mg sinapinic acid equivalents per gram (mg SAE/g).

2.5 Protein analysis

Analysis of protein content was performed using a LECO FP328 Protein analyser (LECO Corp, MI, USA), according to the Association of Official Analytical Chemists (AOAC) method 992.15 (AOAC, 1990). A conversion factor of 6.25 was used to convert total nitrogen to protein.

2.6 Fat and moisture analysis

Total fat was determined using the acid hydrolysis filter bag technique using an automated Ankom Fat Analyser XT15 based on the method ISO 6492.

2.7 Ash analysis

The Ash content of samples was determined by furnace overnight at 600°C following the method of Kolar (1992).

2.8 Angiotensin converting enzyme-I (ACE-I) inhibition assay

Measurement of ACE-I inhibition (EC 3.4.15.1) was performed using the ACE-I kit–WST following the manufacturer's instructions (Dojindo Molecular Technologies Inc., Japan). Briefly, the phenolic extracts and positive control (Captopril®) were assayed in triplicate,

either dissolved in deionized water for the initial assay or 1 % DMSO for the IC_{50} determination. Absorbance was measured using an Omega microplate reader at 450 nm, and % inhibition was calculated using the equation:

[(Absorbance blank 1 – Absorbance inhibitor) / (Absorbance blank 1 – absorbance blank 2)] x 100.

Where, blank 1 is the control without the addition of any inhibitor, blank 2 is the reagent blank, and the inhibitor is the positive control or test sample (extracts).

IC50 was determined by plotting % ACE-I inhibition against concentration (mg/mL) of extract or Captopril, and solving the equation of the line, substituting 50 for the Y value and solving for X.

2.9 Analytical chemical analysis of phenolic extracts

2.9.1 ^{1}H-NMR analysis

^{1}H-NMR spectroscopy was used to quantify the level of sinapinic acid in extract I (previously fraction 3). It was performed using the method of Tan and Shahidi (2013), using Topspin software 2.1 A standard curve was prepared using SA, which was dissolved in Dimethyl sulfoxide-d6 with 1 % (v/v) tetramethylsilane (DMSO-d6 with 1 % TMS (v/v) (Sigma Aldrich, Dublin, Ireland). TMS served as an internal standard for the analysis. Extract I was analysed in

triplicate by dissolving 0.1 g of sample in 1 mL DMSO-d6 containing 1 % (v/v) TMS. In order to calculate the quantity of SA in the fraction, the average values for each standard were plotted in excel and a linear curve generated. The equation of the line was solved, substituting the value obtained for extract I for the Y value and then solving for X.

2.9.2 Mass spectrometry

The extracts and SA were dissolved in acetonitrile: water (+ 0.1 % formic acid) (1:1 v: v) for analysis on the mass spectrometer. The samples were centrifuged at 18000 rcf for 10 min, and the supernatant was removed. The supernatant was then put through a spin column (Thermo scientific, USA) at 2000 rpm (550 x g) for 10 min, and the flow-through was then placed into a vial for analysis.

2.9.3 Thin layer chromatography (TLC)

Silica gel-coated 60G F_{254} TLC plates (Merck, Ireland) were prepared by cutting into 2.5 x 7 cm pieces. Two tanks were prepared to carry out the chromatography. Tank 1 contained 5 mL of ethyl acetate and 5 mL of cyclohexane. Tank 2 contained 10 mL of toluene 2 mL of acetic acid and 1 mL of ethanol. A marker was placed 2 cm up each TLC plate, where the samples were dotted 6 times, and allowed to dry. The plates were then placed into their respective

tanks at an angle to ensure that sample dots do not touch the solvent and the lid placed on top. The plates are removed before the sample dots reach the top of the stationary phase, and allowed to dry. Using ultraviolet light, the spots on the TLC plates were outlined using a pencil. The spots were then visualised by spraying the plates with ferric chloride.

2.10 Enrichment of sinapinic acid content in extract II

To 0.5 g of extract II, generated from the second extraction methodology, 33 mL of deionised water was added. The sample was then mixed by mechanical rotation for 10 min at room temperature, before sonicating for 5 min. The sample was then centrifuged at 5000 rpm (3472 x g) for 10 min, and the supernatant along with excess fat/oil was removed. This process was repeated. The extract was dried at 40 °C, and then ground, ready for use.

2.11 pH analysis of commercial sinapinic acid

Commercial SA was prepared in RPMI 1640 media at a concentration of 4 mM. Both litmus paper and a pH meter (MP230, Mettler Toledo, USA) were then used to measure the pH of SA.

2.12 Cell culture

2.12.1 Cell lines

Cell lines	Origin	Growth	Media	% fetal bovine serum	Additional requirements
HuH 7	Human hepatocarcinoma	Adherent	Eagle's Minimum Essential Medium (EMEM)	10	1 % Penicillin/Streptomycin (P/S) L- glutamine (L-glut)
Hep3B	Human hepatocellular carcinoma (contains Hepatitis B)	Adherent	EMEM	10	1 % P/S 1% L-Glut
HepG2	Human hepatocellular carcinoma	Adherent	EMEM	10	1 % P/S 1% L-Glut
H9c2	Rat cardiomyocyte (model of skeletal muscle)	Adherent	EMEM	10	1 % P/S
SGBS	Human pre-adipocyte	Adherent	Dulbecco's Minimum Essential Medium (DMEM) Ham's F12	10	1 % P/S Panthotenat (17 µM) Biotin (33 µM)

Table 1. Cell lines used in this study along with optimal culturing conditions

Media was warmed to room temperature before use. Cells were cultured in 25 and 75 cm^2 flasks (Corning ®) and maintained at 37 °C, 5 % CO_2.

2.12.2 Cell culture

All cell culture work was performed in a laminar flow cabinet. The unit was switched on at least 15 min before use. The cabinet was wiped down with 70 % industrial methylated spirits (IMS) (v/v) before and after use, while any materials used in the cabinet were sprayed with 70 % IMS (v/v) before entering the cabinet. A lab coat and disposable gloves were worn at all times.

2.12.3 Reconstitution of cell stocks

Cryovials of cell stock suspensions were allowed to rapidly thaw at 37 °C in an incubator. The cells were then transferred using a sterile pasteur pipette into a 25 cm^2 flask (Corning ®) containing 10 mL of pre-warmed complete media. Cells were then maintained at 37 °C, 5 % CO_2.

2.12.4 Preparation of cell stocks

Cells were washed and trypsinised as in section 2.12.5. A volume of 9 mL of complete media was added to each flask, and the cell

suspension transferred to a new flask at the required density for maintenance of the cell line. The remaining cell suspension was centrifuged at 800 rpm (90 x g) for 3 min, after which the supernatant was discarded and the pellet was resuspended in 4 mL of serum-free cryopreservative medium Cell Banker ™ 2 (Zenoaq, Nippon Zenyaku Kogyo Co., Ltd, Japan). A volume of 1 mL was then placed into 4 cryovial tubes, which were placed into the -80 ° C freezer for long-term storage.

2.12.5 Cell line subculture

Cells were passaged once they had reached approximately 80 % confluency. Cell culture reagents were pre-warmed in a 37 °C water bath. Media was removed from the flask and transferred into a waste bottle. The cells were washed with 10 mL Dulbecco's phosphate buffered saline (PBS) (Sigma, USA). The PBS was then discarded into a waste bottle before 1 mL of trypsin ethylene-diamine tetra-acetic acid EDTA (Lonza, Belgium) was added to the flask. The flask was incubated at 37 °C, 5 % $C0_2$ until all of the cells had detached from the flask. The required volume of complete media was added to the flask to neutralize the trypsin. Cells were then transferred to a new flask at the desired density. Cells were maintained in 10 mL complete medium in T75 cm^2 vented flasks at 37 °C humidified air containing 5 % CO_2.

2.12.6 Cell counting

Cell viability was determined using Trypan blue exclusion dye (Sigma, USA). Viable cells have an intact membrane, and so are able to exclude the dye. Dead cells have a permeable membrane and so uptake the dye and appear blue when viewed under the microscope. Cells were trypsinised as described in section 2.12.5. A 50 µL aliquot of cell suspension was mixed with 10 µL of trypan blue. A volume of 10 µL of this mix was placed onto the centre of the upper counting grid on the haemocytometer. A cover slip was then placed over the haemocytometer and viewed at 10x magnification. A total live cell count was obtained for the four outside quadrant grids of the upper counting chamber. The cell number per mL was then calculated as follows:

Cells/ml = (N / 4) x 10^4 x 1.2

N = total live cells counted

4 = the number of grids counted

10^4 = constant

1.2 = dilution factor

2.12.7 Treatments with extracts I, II and commercial SA

Extracts I, II and commercial SA were prepared at the relevant concentrations by dissolving in DMSO: Ethanol (25:75 v: v). The only exception was experiments performed using human peripheral blood mononuclear cells (PBMCs), where extracts I, II and commercial SA were dissolved in complete RPMI-1640 media at the desired concentrations.

2.12.8 Resazurin viability assay

The resazurin assay was performed to determine the viability of cells following treatments with the SA-containing extracts, commercial SA and LPS. Resazurin sodium salt (Sigma, USA) was prepared by adding 0.15 mg/mL to 100 mL PBS (w/v). To perform the assay, 20 µL of resazurin was added per 100 µL of media and incubated for up to 4 hours at 37 °C until a pink colour change was observed. Fluorescence was then measured at excitation / emission = 578 / 604 nm on a Fluoroskan ascent FL plate reader and analysed using Ascent software (Thermo Fisher, USA).

2.13 Reverse transcription polymerase chain reaction

2.13.1 RNA isolation using Tri-reagent

Tri-reagent® (Sigma Aldrich, Ireland) was used to isolate RNA from cell lines. Cells were seeded and treated in 6-well plates for the duration of the treatment period before RNA isolation was carried out. The media was discarded into waste, and 1 ml of Tri-reagent® was added directly into each well. Cells were then scraped from the surface of the well, and transferred to a sterile 1.5 mL eppendorf. If RNA extraction was not performed immediately, samples were stored at -80°C.

To isolate RNA, the samples were thawed on ice. A volume of 100 µl of 1-bromo-3-cholropropane (BCP) was added per 1 mL of cell / Tri-reagent® homogenate and vortexed for 15 seconds. Samples were then centrifuged at 4°C for 15 minutes at 13,500 rcf to ensure phase separation. The top aqueous phase containing RNA was carefully removed into a new 1.5 mL eppendorf, and 500 µl isopropanol was added to precipitate the RNA. The samples were incubated at RT for 5 minutes, and then centrifuged at 13,500 rcf for 8 minutes. The supernatant was discarded and the pellet was washed with 1 mL of 70 % ethanol. The samples were vortexed and centrifuged at 13,400 rcf for 5 minutes, before the ethanol was carefully removed. The pellets were then allowed to air-dry for 5 minutes and re-suspended in 30-50 µl of deionised water depending on the size of the pellet.

2.13.2 RNA quantification

RNA was measured spectrophotometrically using the ND-1000 Nanodrop (Thermo Fisher Scientific, USA). The settings used were 'Nucleic acid' and sample type was 'RNA-40'. The instrument was initialised and a blank measurement made with 1 µl RNase-free molecular grade water on the lower measurement pedestal. A volume of 2 µl of isolated RNA was loaded onto the measurement pedestal. The concentration was recorded in ng/µl. DNA contamination was measured by the 260/280 ratio, with values below 1.8 indicating the presence of DNA. A value above 1.8 indicates the RNA is of acceptable purity.

2.13.3 cDNA synthesis

Removal of any potential genomic contamination was first carried out with the isolated RNA. A volume of sample was taken so that it contained 200 ng of RNA, and made up to a volume of 8 µl with water. A volume of 1 µl of both DNAse and 10 x buffer were then added (Sigma, USA). The samples were then incubated at RT for 15 minutes, before 1 µl of stop solution (50 mM EDTA, Sigma, USA) was added to each sample. The samples were subsequently heated at 70 °C for 10 minutes in the thermal cycler (Fondazione Buzzi Unicem, Italy) in order to inactivate the DNase enzyme.

The samples were then chilled on ice, before 2.75 µl of All-in-One cDNA Synthesis SuperMix (Biotool, Germany) was added to each sample. The reverse transcription reaction was then carried out using the program: 25 °C for 10 min, 42 °C for 1 hr 30 min and 70 °C for 10 min. cDNA was stored at -20 °C.

2.13.4 Polymerase chain reaction (PCR)

The cDNA synthesized (as described in section 2.13.3) was used as the template for RT-PCR. A master mix was prepared up to a final volume of 21 µl, containing the following components per reaction: 10 µl 2x DreamTaq green master mix (Thermo Fisher Scientific, Lithuania), 2 µl forward primer, 2 µl reverse primer, 6 µl Rnase-free water and 1 µl of cDNA. Template cDNA was first denatured at 95 °C for 5 min, followed by 35-40 cycling repeats of 95 °C for 1 min, 60 °C for 1 min (for primer annealing), and extension at 72 °C for 1 min. The cycles were followed by an elongation step of 72 °C for 10 min. Beta-actin and PPIA were used as the endogenous controls. PCR products were stored at 4 °C. The PCR conditions for each primer are listed in Table 2.

The primer sequences for each gene tested were designed by Dr Gray. Briefly, the gene of interest was searched on the OMIM database and the NCBI RefSeq was obtained. In the UCSC website, a BLAT was carried out using the coding sequences in order to map them to the relevant genome. When designing the forward and

reverse primers, regions with approximately 12 guanine and cytosine bases were chosen, without a terminal 3' T and with the resulting amplicon approximately 150 base pairs. The tab "reverse compliment' on the UCSC website was used to generate the sequence of the reverse primer. This sequence, along with that of the forward primer, were pasted into the tab 'In-silico PCR' on the UCSC website and submitted. This displays the melting temperature of the primer pairs, and can be adjusted to the desired temperature by adding bases onto the sequences.

Gene	Sequence	Product size (bp)	Annealing temperature (°C)	Cycles
GLUT 4	**Fwd** :5'-CCCCCTCAGCAGCGAGTGA-3' **Rev** 5'-GCACCGCCAGGACATTGTTG-3'	522	60	40
CXCL 8	**Fwd**:5'-ATGACTTCCAAGCTGGCCGTG-3' **Rev**:5'-ATGACTTCCAAGCTGGCCGTG-3'	186	60	35
TNF-α	**Fwd**: 5'-GATCCCTGACATCTGGAATCTG-3' **Rev**: 5'-GAAACATCTGGAGAGAGGAAGG-3'	129	60	35
β-actin	**Fwd**: 5'TGTTTGAGACCTTCAACACCC 3' **Rev**: 5'AGCACTGTGTTGGCGTACAG 3'	624	60	40
PPIA	**Fwd**: 5'-GGTCCCAAAGACAGCAGAAA-3' **Rev**: 5'-GTCACCACCCTGACACATAAA-3'	115	60	35

Table 2. Primer pairs and PCR conditions used during this project. Each primer was prepared in Rnase-free molecular grade water to a concentration of 5 μM.

2.13.5 Agarose gel electrophoresis

RT-PCR products were analysed by agarose gel electrophoresis. A 2 % (w/v) agarose gel was prepared by adding 4 g of molecular grade agarose (Bioline, USA) to 200 mL of 1 x Tris-acetate EDTA (TAE) buffer (40 mM Tris HCL, 20 mM acetic acid, 1 mM EDTA). The agarose was dissolved in TAE buffer in the microwave until clear. A volume of 25 mL of melted agarose was poured into a container with 4 µl of heat labile ethidium bromide. Combs were then inserted and any bubbles were removed. The gel was allowed to solidify at RT for 15 min, before being submerged in 1x TAE buffer and the combs removed. A volume of 4 µl of GeneRuler 100bp plus DNA ladder (Thermo Fisher Scientific, USA) was added to the first well of each gel. A volume of 10 µl of PCR product was added to each well, except for endogenous control samples which were loaded with 2 µl of product. Electrophoresis was then performed using a GE Lifesciences electrophoresis power supply, with a constant voltage of 80v for 30 min. The gel was visualised under UV light using a Fusion FX chemiluminescence, bioluminescence and UV fluorescence imaging system (Vilber Lourmat Deutschland GmbH, Germany). Gel images were acquired using the Fusion software and saved in .Tiff format for further analysis.

2.13.6 Densitometry analysis

Densitometric analysis was carried out using TINA software. The selected gel image was inverted, with bands selected and analysed for their intensities. The levels of gene expression were calculated as a ratio of sample gene expression to their corresponding endogenous control gene.

2.14 Quantitative polymerase chain reaction (qPCR)

Quantitative polymerase chain reaction was carried out using cDNA reverse transcribed from RNA as a template. A master mix was prepared to a final volume of 21 µl, containing the following components per each reaction: 10 µl 2x Sybr Green qPCR mix, 2 µl of forward primer, 2 µl reverse primer, 6 µl of RNase-free molecular grade water and 1 µl of cDNA. Negative sample wells received only master mix with no cDNA. The samples were added to each well on the 48-well PCR plate and sealed with an adhesive film. The plate was briefly centrifuged to remove air bubbles at 1,300 rpm (235 x g). The qPCR reaction was performed using the Eco™ Illumina machine with Eco software (Illumina, USA), as per cycling conditions described in Table 2.

2.14.1 Quantitative polymerase chain reaction analysis

Data was generated with ECO software (Illumina) following thermal cycling. Relative quantification was then performed using the ΔΔCq

method, with one sample used as a reference for the analysis. In order to normalise the data, PPIA and beta-actin were used as endogenous controls.

2.15 Glucose uptake

Measurement of glucose uptake in the H9c2 cell line was performed using the 2-NBDG Glucose Uptake Assay Kit (Cell-Based) by BioVision (USA). Briefly, H9c2 cells were seeded at a density of 5 x 10^4 cells / well in a 24-well plate. The cells were treated with the phenolic extracts I, II and commercial SA for 2 hours, while the control Phloretin (a known inhibitor of glucose uptake) was added for 1 hour. Uptake was measured on FacsCalibur II machine with 488 nm excitation laser. Data analysis was performed using FlowJo Software, gating on live cells for analysis.

2.16 Western blot

2.16.1 Extraction of histone proteins

Histone proteins were extracted using a method from Abcam with modifications as described. Cells were scraped on ice and washed twice with ice-cold PBS. The cells were then resuspended in Triton Extraction Buffer (TEB) (which consists of PBS with 0.5 % Triton-X and 2 mM PMSF) at a density of 10^7 cells/mL, and lysed by

vortexing on ice every 30 seconds for 10 min. The samples were then centrifuged at 2000 rpm (550 x g) for 4 °C, and the supernatant discarded. The samples were then washed with TEB at half the volume, and centrifuged as before. The pellet was then resuspended in 0.2 M HCL at a density of 4 x 10^7 nuclei/mL, and histone proteins were acid-extracted overnight at 4 °C. The samples were then centrifuged at 2000 rpm (550 x g) at 4 °C for 10 min and the supernatant collected. The supernatant containing the extracted histones was then neutralised with 2 M NaOH at 1/10 the volume of the supernatant. The samples were then stored at – 20 °C.

2.16.2 Bradford protein assay

The protein concentration of the extracted histones were quantified using the Bradford protein assay. A standard curve was prepared using bovine serum albumin (BSA) (Sigma Aldrich, Ireland) dissolved in deionised water ranging from 1500 -0.93 µg/mL. In 1.5 mL Eppendorf tubes, 10 µl of protein sample was mixed with 250 µl of Bradford reagent. Blank samples received just Bradford reagent alone. The samples were mixed for 30 seconds and incubated at room temperature for 15 min. Each sample was then added to its respective wells on a 96-well plate and absorbance was measured at 595 nm using the Fluoroskan Ascent Fl microplate reader (Thermo Fisher, USA).

2.16.3 Preparation of stacking and resolving gels

Two 15 % PAGE gels were prepared as described in Table 3. The resolving gel was prepared as outlined in Table 3, then poured into the rig and topped with a layer of isopropanol. The gel was allowed to solidify at RT for 30 min. The stacking gel was then prepared as described in Table 3. This was poured on top of the resolving gel, combs were inserted, and the stacking gel was then allowed to solidify for 30 min at RT.

Resolving Gel	Volume
4x Separating buffer	5.25 mL
30 % acrylamide	10.5 mL
Water	5.04 mL
TEMED	15 µl
10 % APS	375 µl
Stacking Gel	**Volume**
4 x Stacking buffer	1.75 mL
30 % acrylamide	1.19 mL
Water	3.99 mL
TEMED	7 µl
10 % APS	80 µl

Table 3. Recipes and ingredients to make two 15 % gels for Western Blotting

2.16.4 Gel electrophoresis

The protein samples were prepared to final volume of 75 µl, containing 4x laemmli buffer, molecular grade water and a sample volume containing 10 µg of protein. The samples were then boiled at 95 °C for 5 min before loading onto the gel.

Running buffer (5x) was prepared using: 15g Trizma base, 72 g glycine, and 5 g SDS, made up to 1L with deionised water. A working solution of 1x running buffer was then prepared with deionised water. A volume of 4 µl of protein ladder (Thermo Fisher Scientific) was added to the first well in each gel, while 30 µl of each sample was loaded. Electrophoresis was then performed for 1 hr 30 min at 400 V and 50 mA until the dye front had reached the end of the gel.

2.16.5 Transfer of proteins to PDVF membrane

The gel was placed into a cassette, layered between two sponges and Whatman filter paper. The 0.45 µm PDVF transfer membrane (Thermo Scientific, USA) was activated in methanol for 5 min and then placed directly on top of the gel.

Transfer buffer (5X) was prepared using: 15 g Trizma base and 72 g glycine dissolved in 1 L of water. For the transfer, 1x transfer buffer was prepared by combining 200 mL of transfer buffer (5X), 200 mL

of methanol and 600 mL of water. The proteins were then transferred for 1 hr 15 min at 100 V and 400 mA.

2.16.6 Ponceau S staining

After the protein transfer, 5 mL of Ponceau S solution (Sigma, USA) was added to the membrane and incubated at RT for 10 min with agitation. The Ponceau S solution was then removed using deionised water and the membrane was photographed to determine equal loading.

2.16.7 Detection of acetylated histones

The membrane was washed twice with water, and then blocked for 30 min at room temperature using 3 % non-fat, dry milk in PBS. The primary antibody, anti-acetyl Histone H3 (Upstate Cell Signalling Solutions, USA) was prepared in PBS-milk at a concentration of 0.05 µg/mL. The primary antibody was added to the membrane and incubated at 4 °C overnight. The membrane was washed with water, and then the secondary antibody, a HRP-conjugated goat ant-rabbit IgG (Abcam, UK) was diluted 1:5000 in PBS-milk and incubated for 1.5 hr at room temperature. The membrane was washed with water, and then rinsed with PBS- 0.05 % Tween 20 for 5 min. The membrane was then rinsed with water, and detection was carried out

using Supersignal ® West Pico chemiluminescent substrate (Thermo Scientific, USA).

2.17 Histone deacetylase inhibition assay

Histone deacetylase inhibition was measured using the Epigenase ™ HDAC Activity / Inhibition Direct Assay Kit (Colorimetric) (Epigentek, USA). An acetylated histone HDAC substrate is coated onto the wells, with active HDACs binding to the substrate and removing acetyl groups from the substrate. The HDAC-deacetylated products can then be recognised using a specific antibody, with the activity of the HDAC enzyme proportional to the OD intensity measured. Briefly, the number of strip wells required was determined. The blank wells received assay buffer and HDAC substrate, while the standard wells received assay buffer along with diluted HDAC assay standards. Sample wells received assay buffer, HDAC substrate and histone extracts. Control wells had assay buffer, HDAC substrate and the known HDAC inhibitor TSA. The plate was sealed tightly with an adhesive film and incubated at 37 °C for 90 minutes. The plate was then washed three times with wash buffer, before capture antibody (1:1000) was added, covered in aluminium foil and incubated at room temperature for 30 minutes. The plate was washed again three times using wash buffer. The detection antibody (1:2000) was then added, the plate was covered in aluminium foil and incubated at room temperature for 30 minutes. The plate was washed four times, before developer solution was

added and incubated at room temperature, away from light, for 1-10 min while monitoring colour change. Stop solution was then added and the plate was read at 450 nm using a Biotek ELx808 plate reader. % inhibition was then calculated using the following formula:

$$\textbf{\% inhibition} = 1 - \left[\frac{\text{Inhibitor sample OD} - \text{Blank OD}}{\text{No inhibitor sample OD} - \text{Blank OD}} \right] \times 100\%$$

2.18 Enzyme linked immunosorbent assays

Measurement of TNF-alpha and Il-1beta was performed using the Human TNF-alpha Quantikine ELISA Kit (R&D Systems, Eire), while IL-6, IL-8, and IL-12 were measured using ImmunoTools (Germany). All assays performed were solid phase sandwich ELISA's, where the antigen of interest is sandwiched between a capture and detection antibody. Briefly, capture antibody was added to the plates overnight at room temperature, and then washed using wash buffer (0.05 % Tween 20 in PBS). The plates were then blocked for one hour at room temperature for a minimum of 1 hour using 1 % BSA in PBS. The plates were washed again, before samples (supernatants) were added and incubated for two hours at room temperature. The plates were then washed before detection antibody was added and incubated for 2 hours at room temperature. The plates were washed and streptavidin-HRP was added for 20

minutes. The plates were washed and substrate solution was added for 20 minutes. Stop solution was then added and the plates were read at 450 nm using a Biotek ELx808 plate reader. All washing steps were performed using a Biotek EL. X 405 machine. The data was then analysed using KC Junior software (Biotek, USA).

2.19 Isolation of peripheral blood mononuclear cells from enriched buffy coat

Enriched buffy coat units were obtained from the Irish Blood Transfusion Service at St. James's Hospital, Dublin, Ireland. The enriched buffy coat (25 mL), was placed into a 50 mL tube and diluted 1:1 (v: v) with RPMI-1640 media supplemented with 10 % FBS. A volume of 10 mL of Histopaque® (Sigma Aldrich, Ireland) was added into a fresh 50 mL tube, before 25 mL of diluted sample was slowly layered on top, ensuring the Histopaque® layer was not disturbed. The sample was then centrifuged at 1650 rpm (378 x g) for 25 min, with the brake off, to ensure the resulting layers are not disturbed. The plasma layer was discarded and the buffy coat was carefully removed using a sterile pasteur pipette into a new tube. The cells were then washed by making the volume up to 30 mL with complete media and centrifuging at 2000 rpm (550 x g) for 8 min with the brake on. This process was repeated once more, and the cell pellet was resuspended in a final volume of 45 mL of complete media. A cell count was then performed as described in section 2.12.6.

2.20 Statistical analysis

All data is expressed as mean ± the standard error of the mean (SEM), which represents the standard deviation of the distribution of the sample around the mean. The SEM is calculated as the standard deviation of the original sample divided by the square root of the sample size. Significance was determined by analysis of variance (ANOVA), followed by a post-hoc Dunnett's test. This compares the means of different groups to the mean of a control group. Statistical significance between groups was determined where $P \leq 0.05$. Statistical analysis was performed and graphed using GraphPad Prism 7 (GraphPad software Inc., CA, USA).

Chapter 3

Extraction and Characterisation of Sinapinic Acid from Irish Rapeseed Meal

Data in this chapter forms part of the published work:

'Extraction and quantification of Sinapinic acid from Irish rapeseed meal and assessment of Angiotensin-I converting enzyme (ACE-I) inhibitory activity' Quinn, L *et al*. *J. Agric. Food Chem* **2017** 65 (32), 6886- 6892.

3.1 Introduction

Rapeseed (*Brassica napus*) is one of the world's major oilseeds, and one of the major sources of vegetable oil (Lomascolo *et al.*, 2012). A report published by the EU commission on the trade of rapeseed meal revealed that 448,383 tonnes of meal was exported in 2017/2018, representing a 3% increase on the previous period. Rapeseed meal is a by-product of the de-oiling process and it's most common use is as animal feed. However, rapeseed meal contains bioactive phenolic acids including SA, which could be beneficial for human health. Isolation of this bioactive compound from a by-product of rapeseed oil production is largely in agreement with the current concept of the 'circular economy', whereby resources which would otherwise be considered waste are recycled into the economy, using a biorefinery approach (Velis, 2015). Consumption of a diet rich in fruits and vegetables is known to play a protective role against diseases such as heart disease and diabetes, due to their polyphenol content. However, clinical trials carried out to determine the efficacy of phenolic compounds in the prevention of colorectal cancer revealed the amount required exceeds what is obtainable from dietary intake alone (Nunez-Sanchez *et al.*, 2015). Therefore, one aim of this work was to generate an extract from Irish rapeseed meal containing high-levels of SA. This natural source of SA can then be compared to commercial SA in terms of its bioactive properties.

SA is a 3, 5-dimethoxy-4-hydroxycinnamic acid, which is found in either free or esterified form in rapeseed (Nićiforović and Abramovič, 2014). Phenolic acids in rapeseed meal mainly exist in esterified form, with SA comprising 70-96 % of these esterified phenolics (Nićiforović and Abramovič, 2014). Approximately 15 % of the total phenolics found in rapeseed are free phenolic acids, with SA comprising 65-85 % of free phenolic acids in defatted rapeseed meal (Vuorela, 2005). When determining which method to use in order to extract SA from Irish rapeseed meal, the existing literature was important. There have been no studies published to-date which focused on the extraction of SA from rapeseed meal, rather just the total phenolic content as a whole. This is also true in terms of the bioactive properties of SA. Sinapinic acid derived from a natural source has not been used for *in-vitro* and *in-vivo* studies, with commercial SA used to determine its anti-oxidant, anti-diabetes, anti-cancer and protective roles in cardiovascular disease (Gaspar *et al.*, 2010; Kanchana *et al.*, 2011; Cherng *et al.*, 2013; Silambarasan *et al.*, 2015; Silambarasan *et al.*, 2016; Balaji, Muthukumaran and Nalini, 2015). Given that SA is predominantly found in esterified form, but also exists in free form in rapeseed meal, the extraction methodology of Naczk *et al*. (1992) was chosen. This solvent based methodology allows the extraction and separation of both free and esterified phenolic acids, of which SA comprises the majority of phenolic acids in these fractions.

A novel aspect of this work was to examine the bioactivity of SA isolated from a natural source, Irish rapeseed meal. Polyphenols, including phenolic acids, are commonly found to inhibit the Angiotensin-Converting Enzyme I (ACE-I), which plays a role in hypertension (Al Shukor *et al.*, 2013). The extracts and commercial SA were therefore both tested for their ability to inhibit ACE-I.

Name	**Intervention**	**Primary outcome**	**Status**	**Results**
Postprandial glycemic response to polyphenol-fortified snack bars	Fruit extracts high in polyphenols	Blood glucose concentrations	Completed	Not available
Pharmacokinetic behaviour of phenolic acids derived from black tea	Black tea and phenolic acids	Plasma concentrations of phenolic acids (up to 30 hrs)	Completed	Not available
Effect of phenolic acids on the human vasculature	43.3 mg, 86.6 mg and 173 mg of hydrolysed green coffee extract	Improvement of endothelial function	Completed	Not available

Effects of polyphenolic-rich dark chocolate/cocoa and almonds on cardiovascular disease risk factors	Dark chocolate/cocoa diet; Almond diet; Combination of both diets	Lipid change, 24 hr ambulatory blood pressure, Flow mediated dilation, Lipoprotein class	Completed	Not available
Blueberries for improving vascular endothelial function in post-menopausal women with elevated blood pressure	22 g/day of freeze-dried blueberry powder	Endothelium-based dilation (Baseline and 12 weeks)	Recruiting	N/A
Modulating effects of oil palm phenolics in subjects with pre-diabetes	500 mg gallic acid equivalent, twice daily, 12 months	Blood glucose, serum glycosylated haemoglobin, oral glucose tolerance test	Not yet recruiting	N/A

Table 4. Clinical trials involving the use of phenolic compounds. *(ClinicalTrials.gov)*

3.2 Aims and objectives

The aim of this chapter was to generate and characterise extracts containing the phenolic acid SA, from Irish rapeseed meal.

Specific objectives:

1. Optimise an extraction methodology to prepare extracts containing SA from Irish rapeseed meal
2. Quantify the SA content in the extracts by ^{1}NMR and mass spectrometry
3. Determine the purity of the extracts by Thin layer chromatography (TLC) analysis
4. Determine the initial bioactivity of the extracts by assessing: Angiotensin-converting enzyme-I (ACE-I) inhibitory activity

3.3 Results

3.3.1 Extraction of sinapinic acid from Irish rapeseed meal

Extracts containing sinapinic acid were extracted from Irish rapeseed meal using the method published by Naczk *et al.* (1992), with modifications as described in section 2.3.1. The extraction procedure was repeated and extracts were subsequently pooled together until a sufficient amount, in terms of dry weight, was generated for analysis. Three phenolic extracts were generated using this method: an initial extract (labelled fraction 1), free phenolics (labelled fraction 2) and phenolic acids liberated from esters (labelled fraction 3) (Figure 2).

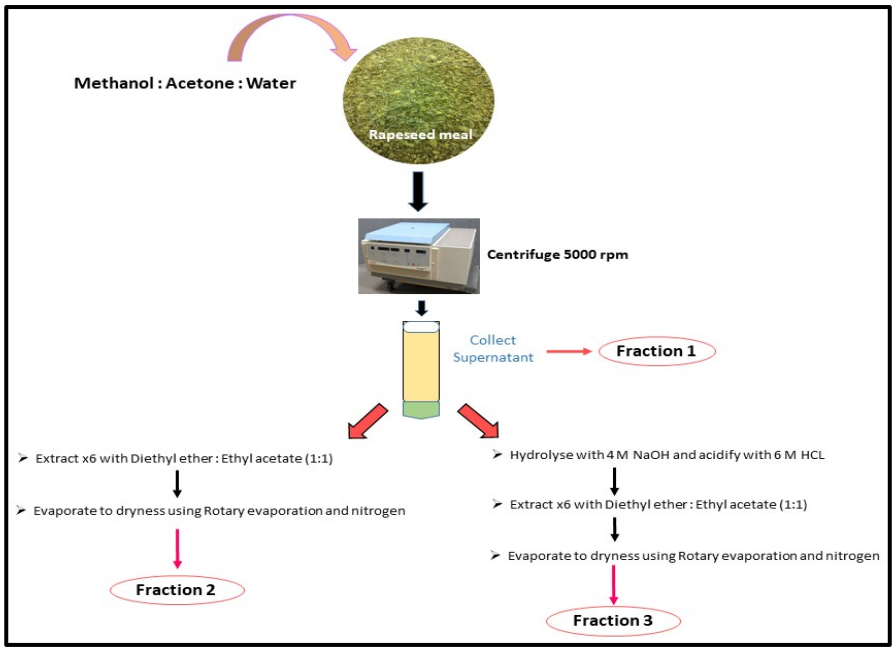


Figure 2. Schematic of the extraction process used to extract SA from Irish rapeseed meal. Three phenolic fractions were generated using a method by Naczk *et al.* (1992), which uses an initial solvent mixture of methanol: acetone: water (7:7:6 v: v: v) to extract phenolics, including SA, from rapeseed meal. The supernatant generated is then separated into two parts, used to generate fractions 2 and 3 as shown above.

3.3.2 Total phenolic content of Irish rapeseed meal phenolic fractions

The total phenolic content of the phenolic fractions generated from Irish rapeseed meal was determined using the Folin-Ciocalteu assay. The assay is based on the transfer of electrons in an alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, which forms a blue complex which can be measured using a spectrophotometer (Ainsworth and Gillespie, 2007). The total phenolic content for each phenolic fraction is shown in Figure 3. The total phenolic content was 200.178 mg SAE/g for fraction 1, 1184.7 mg SAE/g for fraction 2 and 1877.6 mg SAE/g for fraction 3 (Figure 3).

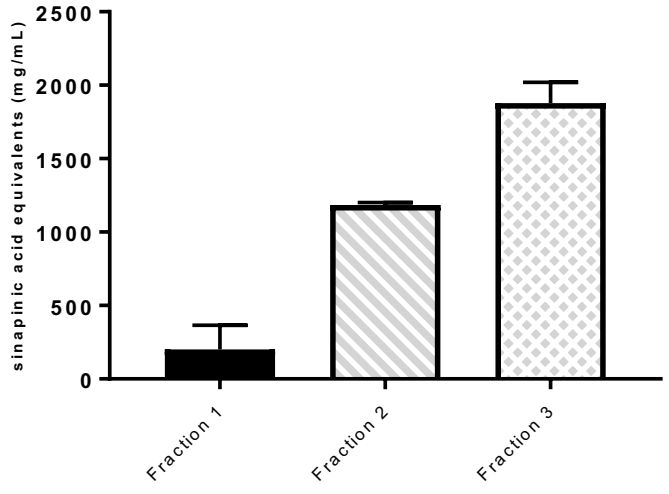


Figure 3. Total phenolic content of Irish rapeseed meal phenolic extracts. Fraction 1 had a TPC of 200.178 mg SAE/g, with 1184.7 mg SAE/g for fraction 2 and 1877.6 mg SAE/g for fraction 3. Analysis was performed using GraphPad Prism 7, and results are displayed as mean ± SEM (*N*=3).

3.3.3 Protein, fat, moisture and ash analysis

Defatting rapeseed meal is an important part of the extraction process given its high oil content. The composition of the extract is also important, especially if the extract is a potential candidate for *in-vivo* studies. Therefore, the protein, fat, and moisture content were determined for fraction 3 (which displayed the highest total phenolic content), along with the extraction source, rapeseed meal, which also underwent ash analysis (Table 5). Total fat was determined by releasing the triglycerides in the sample by acidification with HCL, which were then dried and weighed. Protein in the samples was estimated by multiplying the nitrogen content by a nitrogen-to-protein conversion factor, set at 6.25. Ash content was determined by burning the organic content and leaving inorganic minerals which was weighed and divided by the weight of the original samples. Water was calculated by subtracting the dry weight from the initial weight, with the moisture content calculated as the amount of water divided by the dry weight.

Sample	Protein (N x 6.25)	% Fat	% Ash	% Moisture
Fraction 3	1.6	4.1	Not tested	4.8
Oilseed Rape Meal	3.9	25.7	5.3	10.5

Table 5. Protein, fat, ash and moisture of phenolic fraction 3. The composition of phenolic fraction 3 and rapeseed meal was determined. Rapeseed meal has a high oil content and therefore defatting is a key process. The extract generated has significantly less fat compared to the meal, indicating the defatting process was efficient.

3.3.4 ^{1}H-NMR analysis of extract I (formerly fraction 3)

In order to quantify the amount of SA in our chosen fraction, which his now labelled as extract I, ^{1}H-NMR spectroscopy was performed. Extract I was analysed at a concentration of 0.1g/mL, dissolved in dimethylsulfoxide-d6 (DMSO-d6) (Sigma, USA) with 1% tetramethylsilane (TMS). Commercial SA was prepared using DMSO-d6 with 1 % TMS at various concentrations and used to generate a standard curve (Figure 4 A). The unknown value of extract I was then interpolated from the standard curve, and found to contain 5.31 mg of SA (5.3 %) per 100 mg of fraction (Figure 4 B). This is higher than the values previously reported, which ranged from 0.17 – 1.7 mg/g of SA in rapeseed meal phenolic isolates (Vuorela *et al*. 2004).

(A) (B)

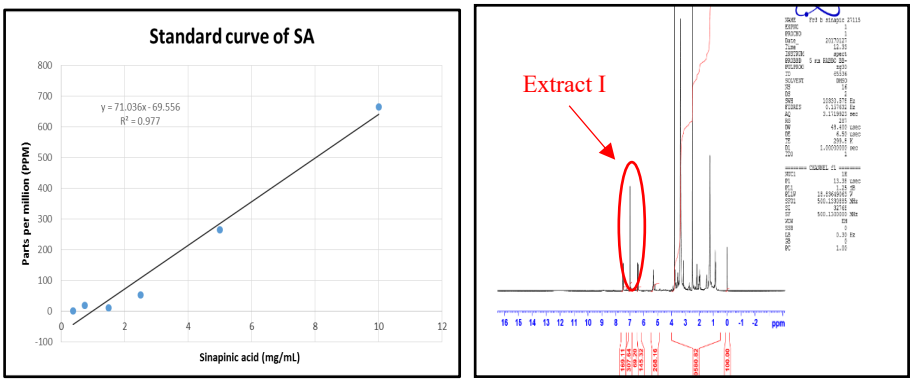


Figure 4. **^{1}H-NMR spectra of extract I**. (**A**) SA was assayed at various concentrations and a standard curve prepared in order to quantify the level of SA in the extract (**B**) Extract I was prepared at a concentration of 0.1g/mL in DMSO-d6 with 1% TMS. TMS served as an internal standard. Analysis was then performed using Topspin software 2.1. The peak for extract I is highlighted by the red circle. Analysis was performed in triplicate.

3.3.5 Modified extraction of sinapinic acid from Irish rapeseed meal

The initial method used to generate extracts containing SA (as described in section 2.3.1), was modified in an attempt to overcome some of the issues faced with the initial extraction, such as a low yield of extract in dry weight, along with a low SA content. These modifications included: improving the yield of extract and SA content, reducing the number of steps involved, and eliminating the use of harmful solvents like diethyl ether.

The modified extraction process is outlined in Figure 5 below. The initial solvent extraction step was removed, with the rapeseed meal directly hydrolysed with 4 M NaOH to release the esterified phenolics in the meal. This is a crucial step given SA is predominantly esterified in rapeseed meal (Vuorela, 2005). The hydrolysed solution is then centrifuged, and the supernatant collected. This is then acidified with concentrated HCL, and extracted using ethyl acetate. After a final centrifugation, the supernatant is then evaporated to dryness (Figure 5).

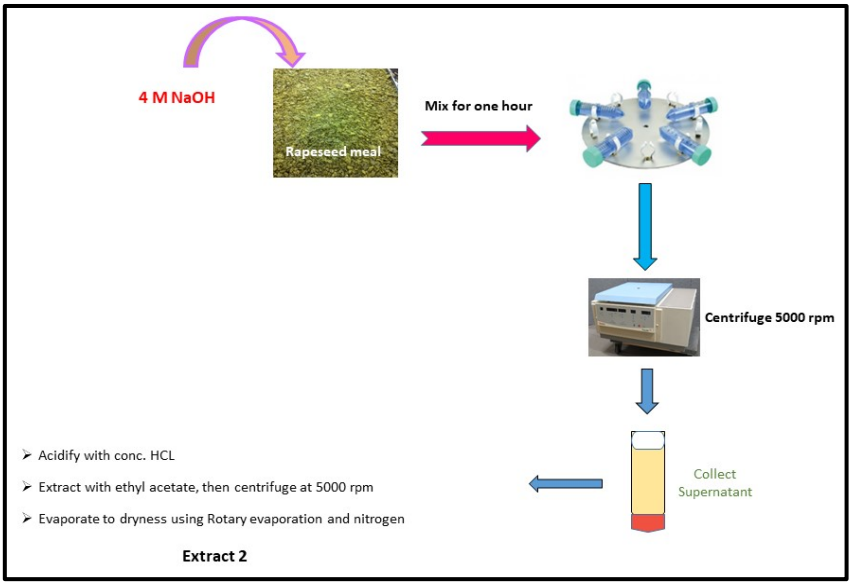
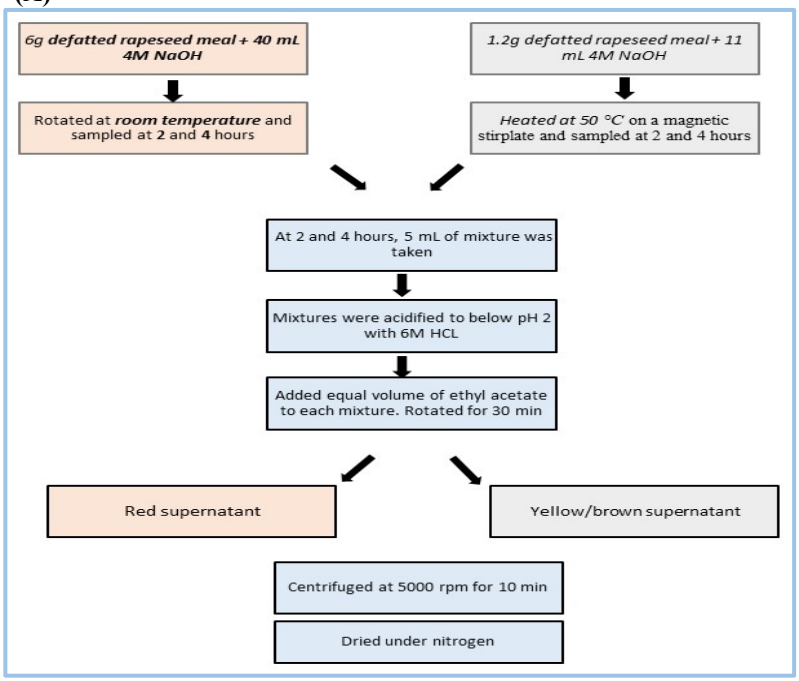


Figure 5. Modified extraction of sinapinic acid from Irish rapeseed meal. Schematic of the modified extraction procedure to generate extract II- containing SA from Irish rapeseed meal.

3.3.6 Time, not temperature, effects SA extraction from Irish rapeseed meal

As part of the modified extraction process, the effect of time and temperature on the yield of SA in the extract was determined. The modified extraction procedure was performed using two different times and temperatures: 2 and 4 hr; room temperature (RT) and 50°C (Figure 6 A). These parameters were applied during the initial step in the extraction process, hydrolysis with 4 M NaOH. Temperature does not affect the yield of SA in the extract, with no significant difference observed between RT and 50 °C at both time points. However, time does affect the yield of SA in extract II. An extraction time of 4 hours significantly reduces the yield of SA compared to 2 hours at RT. There was no significant difference between RT and 50°C at 2 hours, therefore extraction for 2 hours at RT was used to generate extract II (Figure 6 B).

(A)



(B)

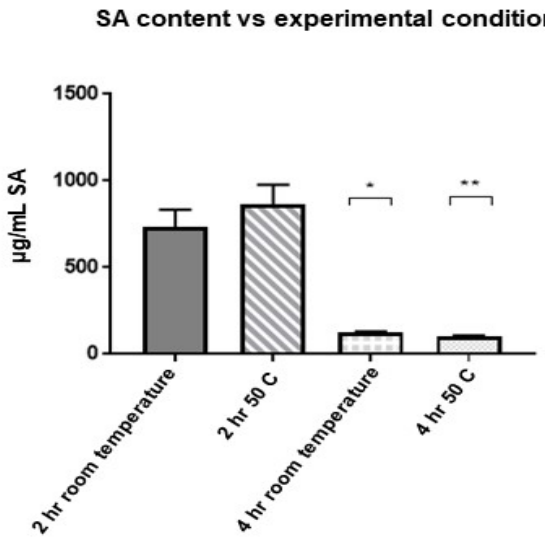


Figure 6. Effect of time and temperature on extraction of SA from Irish rapeseed meal. (**A**) Schematic diagram of the process to test the effect of temperature and time on the yield of SA (**B**) an extraction time of 2 hr at room temperature gives a yield of 734 µg/mL. In comparison, both room temperature and 50 °C after a 4 hr extraction yield significantly less SA, at 124 ug/ml and 102 ug/ml, respectively. Experiments were performed in triplicate and results are displayed as ± SEM, with statistical significance determined for each time-point versus its corresponding temperature ($P < 0.05$).

3.3.7 Thin layer chromatography (TLC) analysis

In order to determine the purity of extracts I and II, thin layer chromatography was carried out with the extracts at concentrations of 1 and 10 mg/mL, while commercial SA was prepared at concentrations of 1 mg/mL, 10 mg/mL and 100 µg/mL. Two tanks were prepared in order to determine the optimal solvent (mobile phase): Tank 1- ethyl acetate: cyclohexane (1:1 v: v); and Tank 2- Toluene (10 mL), acetic acid (2 mL) and ethanol (1 mL). Tank 2 gave a better separation of extracts I, II and commercial SA along the TLC plate. This is highlighted in Figure 7, where commercial SA spotted on the TLC plate in tank 1 has not migrated far from the start line marked on the plate.

The appearance of more than one spot on the plate indicates that there are impurities in the sample. As seen in Figure 7 with tank 2, only one spot appears at each concentration of extract I and II on the plate, which indicates that the extracts are pure.

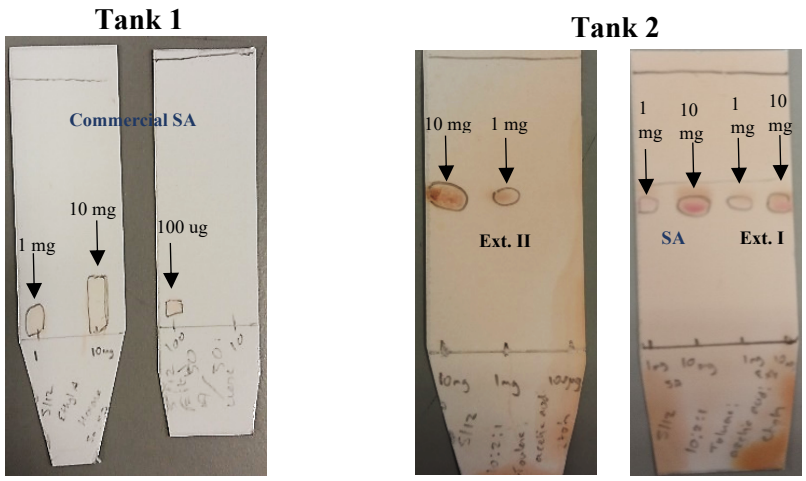


Figure 7. Thin layer chromatography analysis of extracts I and II. Commercial SA was prepared at 1, 10 mg/mL and 100 µg/mL for analysis. Extracts were prepared at 1 and 10 mg/mL. Two different mobile phases were prepared to determine the optimal conditions for the extract. Tank 1 was prepared using: ethyl acetate: cyclohexane (1:1 v: v), while tank 2 contained: 10 mL Toluene, 2 mL acetic acid and 1 mL ethanol. The mobile phase used in tank 2 gave a better separation, as the sample spots migrated further along the plate than those in tank 1. The appearance of one spot at each concentration of extract indicates that they are pure.

3.3.8 Mass spectrometry analysis of extracts I and II

Extract II generated from the modified methodology was analysed for SA content using mass spectrometry. As extract I was analysed using ^{1}H-NMR, its SA content was re-determined using MS for comparison. The extracts were prepared at 1mg/mL using acetonitrile: water (1:1 v: v) with 0.1 % formic acid. Commercial SA was prepared in the same manner at various concentrations to generate a standard curve. It was determined that extract I contained 46.9 µg/mL SA (4.69 %), while extract II contained 90.5 µg/mL SA (9 %) (Figure 8).

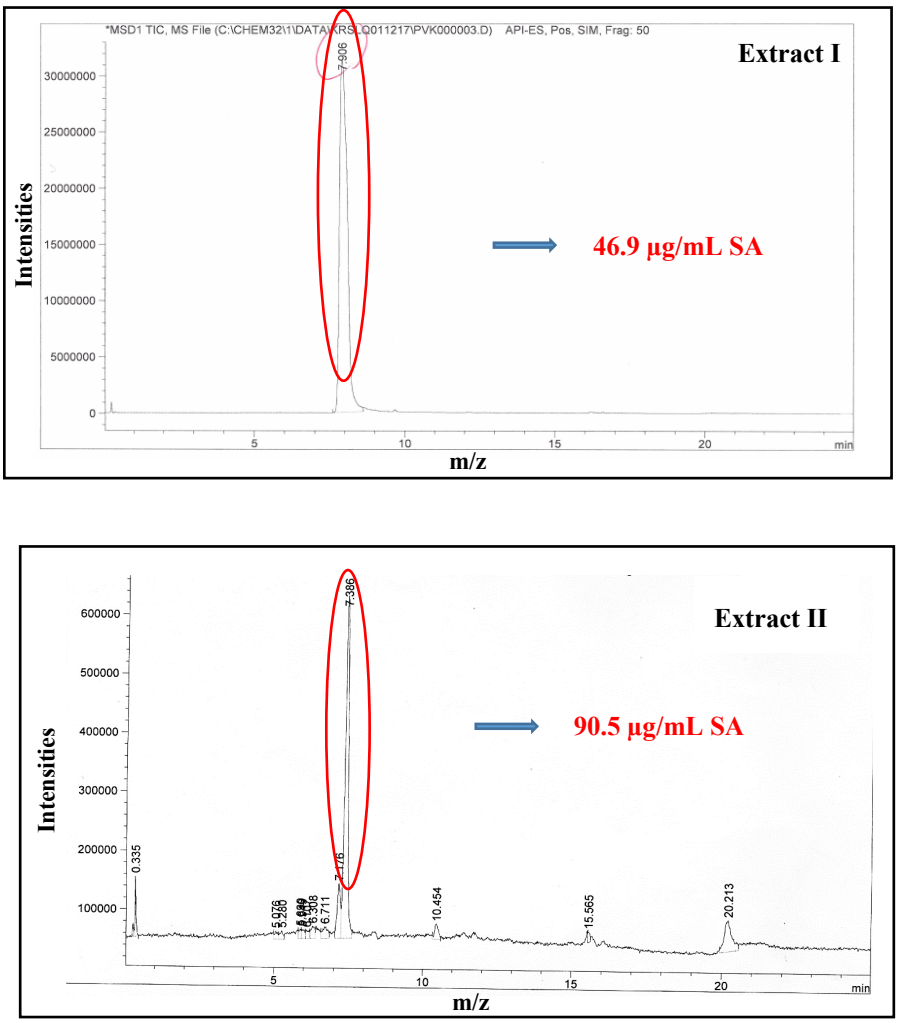


Figure 8. **Mass spectrometry analysis of extracts I and II**. Extracts I and II were analysed at a concentration of 1 mg/mL. SA was identified in the extracts, with retention times of 7.90 and 7. 386 minutes, respectively. The peak areas were then interpolated from the standard curve, and found to contain 46.9 µg/mL and 90.5 µg /mL of SA, respectively.

3.3.9 Enrichment of extract II improves SA yield

While preparing isolated extracts for resuspension using a mortar and pestle, excess oil was noted. Excess oil causes issues when determining the yield of SA, as the oil increases the weight of the extract. This was confirmed by placing the extract on some filter paper and incubating at 50 °C. Leeching of excess oil was then observed on the filter paper beneath the extract. To address this, an enrichment process was applied to extract II using sonication and centrifugation, as described in section 2.10. Deionised water was added to the extract, which was then mixed by mechanical rotation and sonicated. After centrifugation, the oil layer settles on top and can be separated from the extract. This step was repeated in triplicate (Figure 9 A).

In order to ensure that SA is still present throughout this process, samples of extract were taken after the 1st and 3rd wash, and analysed using MS. The MS spectra identified SA in both, with retention times of 7.449 and 7.399 min, respectively. This was confirmed by running commercial SA, which has a retention time of 7.5 min (Figure 9 B).

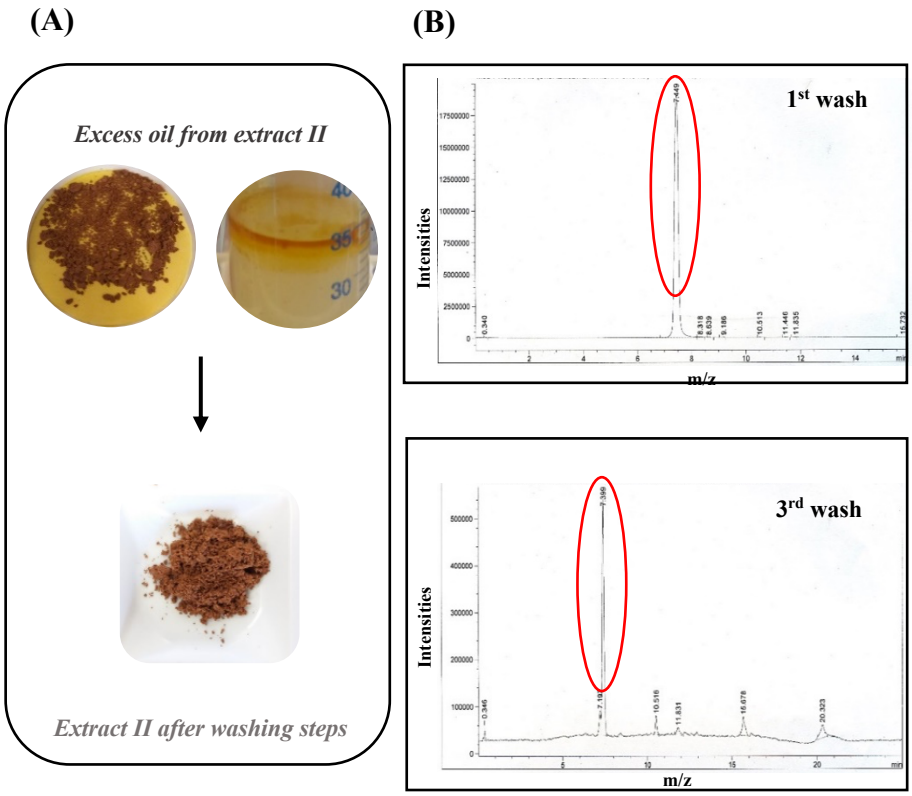


Figure 9. Enrichment of SA in extract II. (**A**) Leeching of oil was observed with extract II, both when placed on filter paper at 50 °C and after washing with deionised water. (**B**) MS analysis was performed with extract taken after the 1st and 3rd wash, to ensure SA is still present. SA was identified in both the 1st and 3rd wash, with the peaks indicated by the red circles. Both the 1st wash (7.449 min) and 3rd wash (7.399 min) had similar retention times to that of commercial SA, at 7.5 min.

3.3.10 Mass spectrometry analysis of enriched extract II

After the enrichment of extract II, MS was performed to quantify the level of SA. Extract II was prepared at 1 mg/mL and diluted 1:400 in acetonitrile: water (1:1) with 0.1 % formic acid. Commercial SA was also ran through MS, and a standard curve generated. SA was detected in extract II, with a retention time of 7.386 min, with commercial SA displaying a retention time of 7.5 min. From the standard curve generated, the enriched extract II was found to contain 0.569 mg/mL of SA, which is a yield of 57 % (Figure 10).

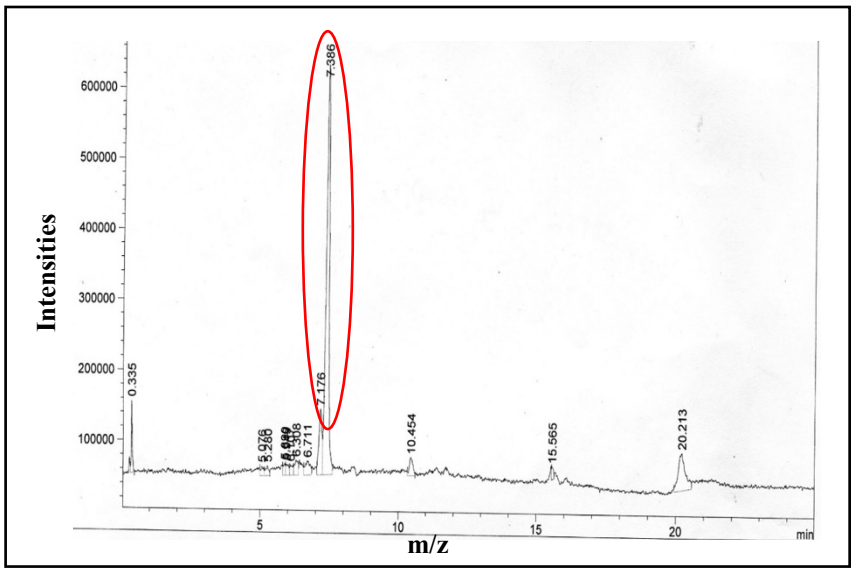


Figure 10. MS analysis of enriched extract II. Extract II was analysed at a concentration of 1 mg/mL, diluted 1:400. SA was identified in the enriched extract II, with a retention time of 7.386 minutes (highlighted in red). The peak area was then interpolated from the standard curve, and found to contain 0.569 mg/mL of SA.

3.3.11 Sinapinic acid content of rapeseed meal phenolic extracts I and II

Sinapinic acid was the main phenolic acid of interest when generating extracts I (formerly named fraction 3) and II (now enriched) from rapeseed meal. In all experimental work, commercial SA was tested alongside the extracts. It was therefore necessary to calculate the sinapinic acid content in each extract. The extracts were tested at concentrations ranging from 1-0.0625 mg/mL, and so the SA content at each of these concentrations was determined (Table 6). This also allows for relevant concentrations of SA to be tested for comparison to the generated extracts.

Concentration (mg/mL)	**Extract I (equivalent SA content in mM)**	**Extract II (equivalent SA content in mM)**
1	0.472	5
0.5	0.236	2.5
0.25	0.118	1.25
0.125	0.059	0.625
0.0625	0.029	0.312

Table 6. The SA content of extract I and II at each of the concentrations used for the experimental work.

3.3.12 ACE-I activity

ACE-I is a zinc-dependent peptidase which cleaves angiotensin I to angiotensin II, which is a vasoconstrictor involved in regulating blood pressure (Bernstein *et al.*, 2013). Recent evidence suggests that various processes including oxidative stress, inflammation and hypertension are interconnected, with each influencing the other in terms of the pathology of 'hypertension-related cardiovascular events'. Therefore, developing inhibitors which can target ACE-I and which also possess anti-oxidant activity are of great interest (Pacurari *et al.*, 2014). Given that current ACE-I inhibitors such as Captopril® and Enalapril® have various side effects including cough, rash and vomiting, identifying ACE-I inhibitors from a natural food source is an area of interest (Sharma, 2015).

The three phenolic fractions generated (total, free and esterified) were tested for their ability to inhibit ACE-I, compared to a positive control Captopril. All three fractions were found to inhibit ACE-I, with 91 ± 3.6, 89 ± 2.2 and 90 ± 0.3 % inhibition for esterified, total and free phenolics, respectively. This is similar to Captopril, which displayed 96 ± 0.5 % inhibition (Figure 11 A).

Extract II (enriched), which was generated using the modified extraction method, along with commercial SA, were tested for ACE-I inhibition. Extract II was tested at both 1 and 0.5 mg/mL, with 97 ± 4.1 and 88 ± 2.6 % inhibition, respectively. The positive control Captopril displayed 100 ± 1.5 % inhibition. When commercial SA

was tested at 5 mM, it displayed 48 ± 16.4 % ACE-I inhibition (Figure 11 B).

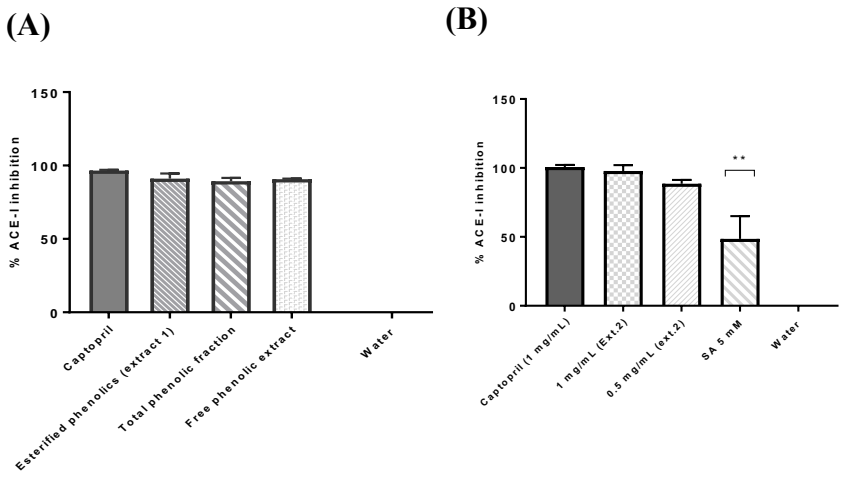


Figure 11. Assessment of ACE-I inhibitory activity. (**A**) The extracts generated using the initial methodology, total, free and esterified phenolic acids (labelled extract I), were tested at 1 mg/mL for their ability to inhibit the ACE-I enzyme. All three fractions inhibited ACE-I, with 91, 89, 90 % inhibition for esterified, total and free phenolics, respectively. This is similar to the positive control Captopril, which displayed 96 % ACE-I inhibition (*N*=3) (**B**) Enriched extract II, generated using the modified extraction method, along with commercial SA, were tested for ACE-I inhibition. Extract II was tested at both 1 and 0.5 mg/mL, with 97 and 88 % inhibition, respectively. This was similar to the positive control Captopril, which displayed 100 % inhibition. It appears that commercial SA is not as effective as either extract I or II, displaying 48 % ACE-I inhibition (*N*=3). Statistical analysis was performed using Dunnett's test (vs Captopril, $p < 0.05$).

3.4 Discussion

Despite being viewed as a by-product which is not suitable for human consumption, rapeseed meal contains bioactive phenolics with potential health benefits (Vuorela, 2005). As discussed, clinical trials have shown that the amount of phenolic acids required to exert their effects exceeds what is obtainable from dietary intake (Nunez-Sanchez *et al.*, 2015). Therefore, extracting phenolics such as SA from rapeseed meal could enhance the economic value of this resource, and provide an extract with potential use as a functional food ingredient. This also encompasses the idea of a 'circular economy', where resources which are considered waste are recycled back into the economy (Velis, 2015).

There is no existing methodology in which SA is isolated from rapeseed meal, rather total phenolics are isolated and characterised. As such, a method for extracting different phenolic fractions was chosen based on the phenolic composition of rapeseed meal. Phenolic compounds in rapeseed mainly exist in esterified form, bound to other phenolics, sugars or kaempferols. SA comprises 70-96 % of esterified phenolics, while SA constitutes 65-85 % of the free phenolics (Naczk, Wanasundara and Shahidi, 1992). The method of Naczk *et al*. (1992) allows for the extraction of both free and esterified phenolics, thereby allowing the greatest possible yield of SA. Once each fraction was generated, they were characterised in terms of their total phenolic content (TPC), fat, moisture and ash content. Fraction 3, containing the soluble esters, had the highest

total phenolic content. It has previously been shown that esterified phenolics appear to be extracted more effectively than free phenolic acids, as is the case in this work. However, no explanation has been proposed for this observation (Naczk, Wanasundara and Shahidi, 1992).

There were some issues when generating the three phenolic extracts. In order to generate the free and esterified phenolics, the initial supernatant generated must be divided into two parts. This therefore reduces the yield of each fraction in terms of dry weight. As such, multiple extractions were performed in order to generate enough of each fraction for further characterisation. It was decided to attempt to modify this method, in order to produce a greater amount of extract, along with reducing the number of steps and solvents used.

The majority of phenolic acids found in rapeseed meal are esterified, and the results have shown that fraction 3 containing soluble esters (renamed as extract I moving forward), had the highest TPC and highest level of ACE-I inhibition. As such, it was decided to focus on extracting the esterified phenolics from the meal in order to extract SA. One of the main phenolics in rapeseed meal is Sinapine (the choline ester of sinapinic acid), and hydrolysis of sinapine yields SA. Rather than use a solvent mix to generate a phenolic extract which is then hydrolysed, the rapeseed meal itself was hydrolysed with NaOH. Acid/alkali hydrolysis is the most common method used to release phenolic compounds by breaking ether and ester bonds

(Irakli *et al.*, 2018). This reduces the number of solvents used, and eliminates a step which produces an extract with a low total phenolic content and low SA content. A larger volume of rapeseed meal could be used in one extraction procedure, given there is no longer a need for repeated solvent extractions. The supernatant generated can then be directly hydrolysed to release the esterified phenolics, producing a greater yield of extract in terms of dry weight in one extraction procedure. There is variation in the literature with regards to the temperature and time used for extracting phenolic acids, (Vuorela, Meyer and Heinonen, 2003; Khattab *et al.*, 2010; Cai and Arntfield, 2001). To ensure optimal extraction conditions, both time and temperature were assessed in terms of their effect on the yield of SA. Based on these results, it seems time is a crucial factor affecting SA yield. At both room temperature and 50 $^{\circ}$C, hydrolysis for 4 hours significantly reduced the yield of SA compared to a 2 hour extraction. Prolonged treatment in alkaline conditions can lead to degradation of the phenolic acids which have been released. A recent study which aimed to optimise the extraction of free and bound phenolics from rice by-products found that 120 min was the optimal length of time to extract the bound phenolic acids (Irakli *et al.*, 2018). This mirrors the results in this chapter, with 120 min of alkaline hydrolysis giving the best yield of SA from rapeseed meal.

The SA content of extract I was determined previously using ^{1}H-NMR spectroscopy. It was decided to re-analyse extract I using MS along with extract II. Mass spectrometry analysis determined that

extract I contained 46.9 µg/mL SA (4.69% w: v). This is similar to the SA content previously quantified using ^{1}H-NMR, which was found to be 53 µg/mL (5.3 % w: v). Extract II had an SA content of 90.5 µg/mL or 9% (w: v), indicating the modified methodology improves the yield of SA. In order to assess the purity of the extracts, TLC analysis was performed. The results indicate that the extracts are pure, as only 'spot' appears on the plate at each concentration tested.

It was noted that extract II still retained some oil, which was evident by the leeching of oil when the extract was dried on filter paper at 50°C. As such, further steps were performed to try to remove as much of this as possible. After washing and sonicating the extract with water, the oil layer settled on top and could be removed effectively after three washings. MS analysis was then carried out, which showed that the yield of SA had increased to 57 %. This is likely due to the fact that the dry weight of the extract is reduced and more concentrated, thereby increasing the amount of SA in this final extract II.

The main aim of this chapter was to generate an extract containing the bioactive phenolic acid SA from Irish rapeseed meal, with potential value for improving human health. Therefore, preliminary experiments were carried out to determine if the extracts possessed potential bioactivity to improve heart health. ACE-I is a zinc-dependent peptidase which cleaves angiotensin I to angiotensin II,

which is a vasoconstrictor involved in regulating blood pressure (Bernstein *et al.*, 2013). Angiotensin II has many effects, including the vasoconstriction of precapillary arterioles and postcapillary venules, along with promotion of hypertrophy of vascular smooth muscle cells and cardiac myocytes. As such, along with hypertension, ACE-I inhibitors are indicated for heart failure, as less myocyte hypertrophy is observed (Herman and Bashir, 2019). An extract with ACE-I inhibitory activity could therefore be an important, natural alternative to chemically synthesised inhibitors. Both phenolic extracts I and II, generated from Irish rapeseed meal, proved to be effective ACE-I inhibitors, with their inhibition similar to Captopril, which is currently used in the clinical setting. Both extracts also displayed superior ACE-I inhibition compared to commercial SA alone. One explanation for this observation could be due to the fact that the extracts generated do not contain 100 % SA, and it is likely there are other phenolic acids present. Other minor phenolic acids found in rapeseed which could be present in the extracts include: vanillic, syringic, *p*-coumaric, ferulic and caffeic acids (Khattab *et al.*, 2010). All of these phenolic acids have been shown to inhibit ACE-I in-vitro, with ferulic acid also shown to lower blood-pressure *in-vivo* (Al Shukor *et al.*, 2013). It has been suggested that phenolic acids, including hydroxycinnamic acids such as sinapinic, p-coumaric, ferulic and caffeic acids, display higher ACE-I inhibition with increasing numbers of hydroxyl groups. These functional groups act as hydrogen-bond acceptors or

donors, and those phenolics with a lower number of hydrogen-bond acceptor groups displayed lower ACE-I inhibition (Al Shukor *et al.*, 2013).

A naturally occurring ACE-I inhibitor could also be useful in terms of reduced toxicities. All ACE-I inhibitors with the exception of 2, are prodrugs which require activation in the liver. This is not necessary with the extracts generated, and therefore would be suitable for those with impaired hepatic function (Herman and Bashir, 2019). Current ACE-I inhibitors are taken orally, which would be the likely route of ingestion with a bioactive extract containing SA. Indeed, the small intestine has been reported as the optimal site for absorption of orally administered SA, through active NA^+ gradient transport. SA is then also metabolised via phase I and II reactions in the small intestine (Chen, 2016).

The overall objective of this chapter was to generate an extract containing SA from Irish rapeseed meal with bioactivity. This aim was achieved, however there are some issues with this work which could be addressed. It would be beneficial to determine if other phenolic acids are present within the extract. This could be done by purchasing standards of various phenolic acids, and running them through the mass spectrometer alongside our extracts. However, a recent study by Huang *et al*. (2019) found that the optimal method for separating and identifying phenolic acids in rapeseed extracts was by capillary electrophoresis. This method could be used to

identify the phenolics in the extracts, and aid in elucidating the components contributing to the observed bioactivity.

Chapter 4

Assessment of the biological activities of rapeseed meal extracts I, II and commercial sinapinic acid: Type II diabetes mellitus

4.1 Introduction

The term ‘metabolic syndrome’ is characterised by a combination of risk factors occurring simultaneously, which include: central obesity, insulin resistance, dyslipidaemia and hypertension. Their cumulative effects lead to an increased risk of type 2 diabetes (T2DM) and cardiovascular disease (O'Neill and O'Driscoll, 2015). Diabetes mellitus is a ‘complex metabolic disorder’, whose main clinical feature is hyperglycemia (Zaccardi *et al.*, 2016). Diabetes affects approximately 387 million people worldwide, with its prevalence expected to double within the next 20 years (Zaccardi *et al.*, 2016). Type II diabetes mellitus, also termed ‘non-insulin-dependent’, is steadily increasing, with Western-style lifestyle habits including lack of physical activity, sedentary behaviour and poor diet established as risk factors in disease development (Zaccardi *et al.*, 2016). Type II diabetes is characterised by insulin resistance, which results in impaired glucose uptake into muscle and adipose tissues, along with high circulating plasma glucose levels (Bryant et al. 2002). Those suffering from T2DM also have an increased risk of developing cardiovascular disease, cancer, liver disease and infections (Zaccardi *et al.*, 2016). The global costs involved in the clinical management of diabetes and its co-morbidities are expected to considerably increase by 2030. It is predicted that the global economic burden of T2DM will not decrease, regardless of individual countries abilities to meet international targets (Bommer *et al.*, 2018).

Glucose is a vital energy source for all eukaryotic cells. Once digested and absorbed in the gut, glucose must be distributed to the various tissues in the body. This is achieved through a family of glucose transporters called 'GLUT's', of which GLUT4 is a key member, particularly in relation to the development of T2DM (Bryant, Govers and James, 2002). GLUT4 is primarily found in muscle and fat cells, and is responsible for lowering plasma glucose levels. Upon stimulation by insulin, GLUT4 translocates from an intracellular position to the cell surface, where glucose uptake occurs (Bryant, Govers and James, 2002). GLUT4 is also believed to play an important role in whole-body glucose homeostasis, as insulin-stimulated glucose transport is a key rate-limiting step in glucose metabolism in fat and muscle tissues, and is severely disrupted in T2DM. Furthermore, *in-vivo* studies have shown that impaired GLUT4 expression in mice leads to insulin resistance, which is associated with a loss of insulin sensitivity (Bryant, Govers and James, 2002). A study by O'Byrne *et al*. (2011) also found that GLUT4 is epigenetically regulated, with treatments with Trichostatin A (TSA), a known inhibitor of HDACs, inducing GLUT4 expression in liver, pre-adipocyte and skeletal muscle cells (O'Byrne *et al.*, 2011). As such, drugs or natural bioactives that can increase insulin sensitivity by affecting levels of GLUT4 could have potential impact.

One of the most crucial factors underlying the pathogenesis of metabolic diseases is obesity. Adipose tissue releases non-esterified

fatty acids (NEFAs), along with glycerol, hormones such as leptin and adiponectin and pro-inflammatory cytokines (Kahn, Hull and Utzschneider, 2006). It is the release of NEFAs which is crucial when it comes to insulin sensitivity, as increased levels of NEFAs are present in type II diabetes and obesity, both of which are associated with insulin resistance. An increase in plasma NEFA levels leads to the development of insulin resistance within a few hours in humans, as they inhibit insulin-stimulated glucose uptake and glycogen synthesis. This highlights the key role NEFAs play in modulating insulin resistance (Kahn, Hull and Utzschneider, 2006).

An area of increasing interest is the use of natural polyphenols to treat diseases such as T2DM, with numerous studies, both *in-vitro* and *in-vivo*, published in recent years (Zheng *et al.*, 2018). The anti-diabetic activity of a polyphenol-rich extract from *Phellinus igniarius* (PI-PRE) has recently been characterised in KK-AY mice, which serve as an experimental model of T2DM. Mice treated with PI-PRE had a decrease in fasting blood glucose levels, while GLUT4 expression was increased in skeletal muscle (Zheng *et al.*, 2018). Similar results were obtained with the herbal tea *Kinkeliba,* which contains bioactive polyphenols, in C57BL/6J mice. This *in-vivo* study also lowered plasma glucose in a dose-dependent manner without significant toxicity (Welch *et al.*, 2018).

A study published this year examined the effects of a polyphenol clove extract (PCE). Volunteers, both healthy and pre-diabetic, were

given PCE supplements at 250 mg concentrations once a day for 30 days. Supplementation with the extract was found to lower pre and post prandial blood glucose levels in both healthy and pre-diabetic volunteers, with a significant reduction observed after 12 days (Mohan *et al.*, 2019).

As SA is postulated to affect epigenetically regulated genes, we hypothesise that SA-containing extracts could potentially affect levels of GLUT4 and/or affect other pathways linked to diabetes/obesity. Therefore, the objective of this chapter was to determine the potential anti-diabetic effects of phenolic extracts I and II generated from Irish rapeseed meal.

4.2 Aims and objectives

The aim of this chapter was to examine the effects of the generated extracts I and II on various cell lines in order to determine a non-toxic dose. Once this was determined, the extracts, along with commercial SA (control), were tested to determine their potential to ameliorate T2DM.

Specific objectives:

1. Determine the effect of commercial SA on histone deacetylase expression in the H9c2 cell line
2. Determine a non-toxic dose of extracts I and II, along with commercial SA in SGBS, H9c2, HuH7, HepG2, Hep3B and Caco-2 cell lines.
3. Determine the effects of phenolic extracts I, II and commercial SA on GLUT4 expression in the H9c2, HuH7 and SGBS cell lines.
4. Determine the effects of extracts I and II, along with commercial SA, on glucose uptake in the H9c2 cell line.

4.3 Results

4.3.1 Commercial sinapinic acid displays histone deacetylase inhibitory activity

Histone deacetylase inhibitors are known to play important roles in inflammation, diabetes and insulin sensitivity. One hypothesis regarding the potential bioactivity of SA and extracts containing SA, is based on the ability of SA to act as a histone deacetylase inhibitor (HDACi). Only one study by Senawong *et al*. (2013) has shown that SA can inhibit histone deacetylase activity, with an IC_{50} of 2.27 mM.

Commercial SA was tested for HDACi activity at 3 and 24 hours using a HDACi assay kit. SA was tested at concentrations ranging from 2.2 to 10 mM in both the HuH7 and H9c2 cell lines, given previous work by O'Byrne *et al*. (2013) found that GLUT4 is epigenetically regulated in these cell lines. After 3 hr treatments with SA, there was no effect on HDACi activity in both the H9c2 and HuH7 cell lines (Figure 12 A and B). However, following 24 hour treatments with SA, 10 mM and 20 mM significantly reduced HDACi activity in the H9c2 cell line, while 10 mM also significantly inhibited HDACi activity in the HuH7 cell line. Treatment with SA at 2.2 and 4.46 mM for 24 hours did not show any inhibition of HDACi activity (Figure 12 C and D).

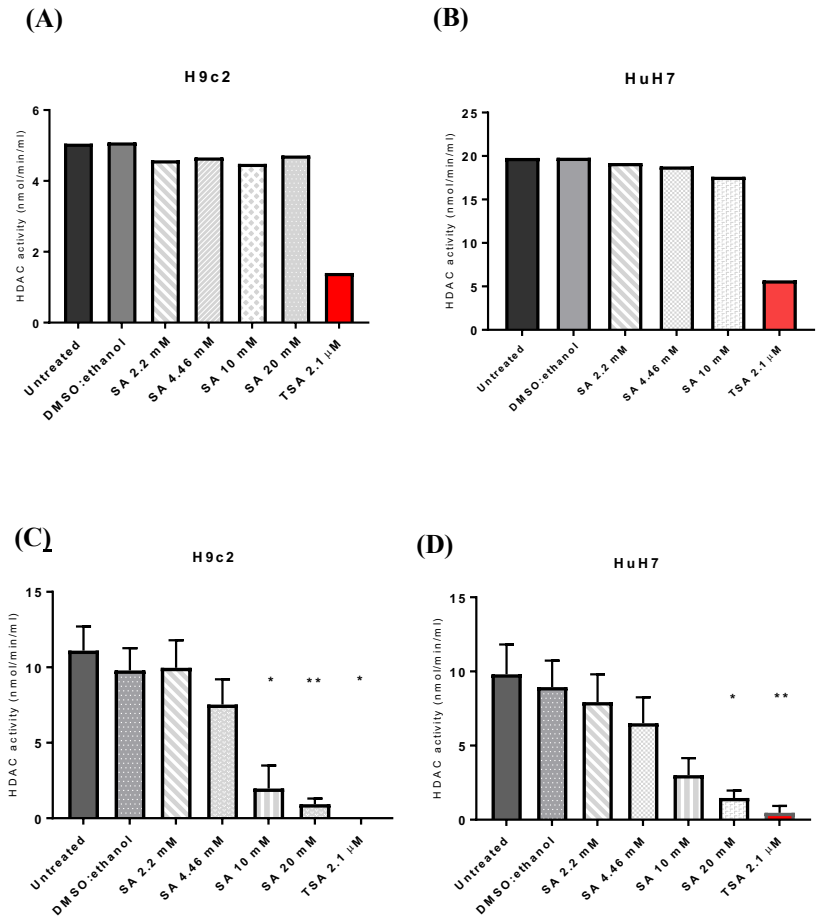


Figure 12. Assessment of histone deacetylase inhibitory activity of SA. (**A**) 3 hour treatments with SA do not effect HDACi activity in the H9c2 cell line (**B**) 3 hour treatments with SA do not effect HDACi activity in the HuH7 cell line (**C**) 24 hr treatments with SA significantly inhibits HDAC activity at concentrations of 10 and 20 mM (*N*=3). (**D**) 24 hour treatments with SA at 20 mM in the HuH7 cell line significantly inhibits HDAC activity. (*N*=3). Statistical analysis was performed using a post-hoc Dunnett's test (vs control; $P < 0.05$), results are displayed as ± SEM.

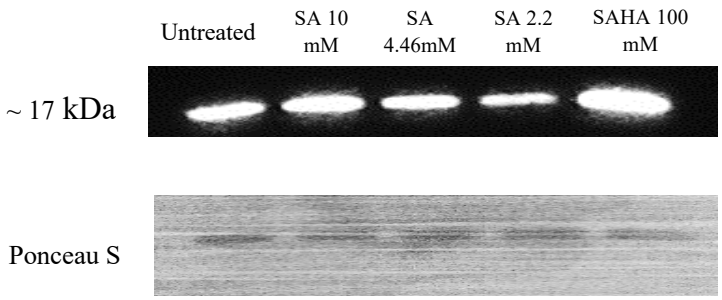
4.3.1.1 Commercial sinapinic acid does not inhibit histone deacetylase activity in the H9c2 cell line

The hypothesis for commercial SA's bioactivity stemmed from its function as a histone deacetylase inhibitor. Previous work by Senawong *et al*. (2013) demonstrated that SA acts as a HDACi, with an IC_{50} of approximately 2.2 mM. HDAC inhibitors were shown previously to influence GLUT4 expression. Data from the HDACi assay in section 4.3.1, demonstrated that SA can inhibit HDACs by ELISA analysis. Therefore Western Blot analysis was performed in order to confirm or deny this.

At both 3 and 24 hour time-points, the positive control Vorinostat/SAHA (Suberanilohydroxamic acid) shows an increase in band density, indicating increased acetylation which is consistent with its known function as a HDACi.

At selected time-points of 3 and 24 hours, SA at a concentration of 2.2 mM appears to cause a decrease in histone acetylation, as evidenced by the decrease in band size. At 24 hours, SA at a concentration of 4.4 mM also showed a decrease in band size. These results indicate that SA does not act as a HDACi (Figure 13).

3 hours



24 hours

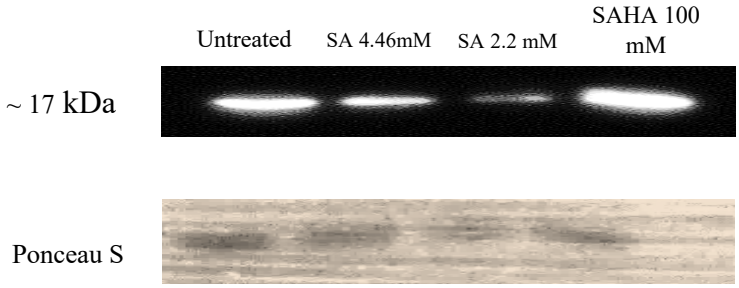


Figure 13. Western Blot analysis of histone H3 acetylation following treatments with commercial SA. Histone proteins (~17 kDa) were extracted from H9c2 cells after 3 and 24 hr treatments with SA and positive control SAHA (a known HDAC inhibitor). The histone proteins (10 µg) were probed with anti-acetyl H3 antibody at 0.05 µg/mL, and detection was carried out using Supersignal® West Pico chemiluminescent substrate. Equal loading was confirmed by Ponceau S staining of each membrane following protein transfer. **3 hours**: SA decreased acetylation at a concentration of 2.2 mM. **24 hours**: At concentrations of 2.2 and 4.4 mM, SA decreased acetylation. These results indicate that SA does not act as a histone deacetylase inhibitor. Experiments were performed using three biological replicates. *N*=3.

4.3.2 Toxicity profile of rapeseed phenolic extract I and sinapinic acid

The resazurin cellular viability assay was performed in order to determine a non-toxic concentration of phenolic extract I and commercial SA for use in downstream studies. Initially, three cell lines were chosen: pre-adipocytes (SGBS), liver (HuH7) and muscle (H9c2) cells. These cell lines are relevant for studies to determine the potential anti-diabetic effects of the extracts, given muscle and adipose tissue are the main sites of GLUT4 expression, while a study by O'Byrne et al. (2011) has shown that GLUT4 is epigenetically regulated in the H9c2 cell line.

Both phenolic extract I and commercial SA were shown to be non-toxic in the SGBS cell line. However, both the HuH7 and H9c2 cell lines had a significant decrease in viability when phenolic extract I was applied at a concentration of 1 mg/mL ($p < 0.05$), with negligible effects on toxicity at concentrations below 0.25 mg/mL (Figure 14). A concentration of extract I of 0.25 mg/mL was chosen for use in downstream studies.

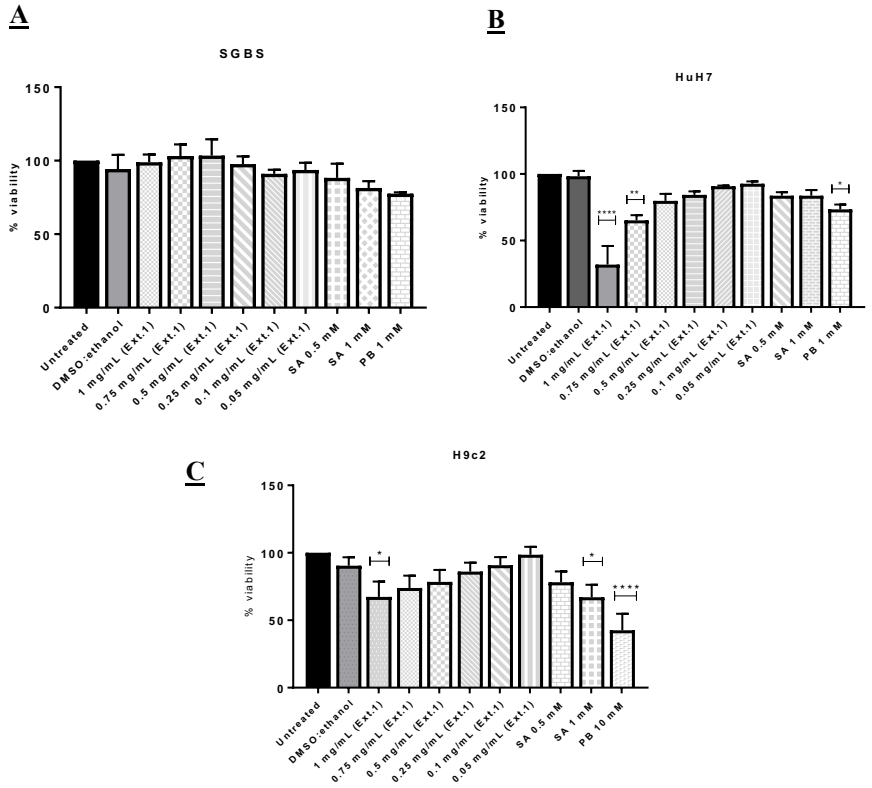


Figure 14. Cellular viability of SGBS, H9c2 and HuH7 cells following 48 hour treatments with extract I and commercial SA. The resazurin viability assay was performed to assess cellular viability (**A**) There was no significant differences in the viability of SGBS cells after treatments with extract I and commercial SA cell (**B**) Extract I at a concentration of 1 mg/mL and 0.75 mg/mL significantly reduced the viability of HuH7 cells, while commercial SA did not have any significant effects (**C**) Extract I at a concentration of 1 mg/mL significantly reduced the viability of H9c2 cells, while commercial SA also significantly reduced viability at 1 mM. Experiments were performed in biological triplicate. Statistical analysis was performed using a one-way ANOVA with a post-hoc Dunnett's test, vs control. Control sample is DMSO: Ethanol (25:75 v: v). Results are displayed as ± SEM, with statistical significance where $p < 0.05$. *N*=3.

4.3.2.1 Effect of commercial sinapinic acid on the viability of liver cell lines

The effect of commercial SA was determined using a range of concentrations and time-points in three liver cell lines - Hep3B, HepG2 and HuH7. These cell lines are commonly used in toxicity screens, as well as in studies involving obesity and diabetes. The equivalent SA content in mM was determined for both extracts I and II, as seen in table 7. The highest equivalent concentration was 5 mM and the lowest 0.3 mM, therefore commercial SA was tested for its effects on cell viability at 0.4, 3.3 and 4 mM.

In the Hep3B cell line, at time-points of 2, 4 and 24 hours, SA at 3.3 and 4 mM significantly reduced cellular viability. At 2 hours, SA at a concentration of 0.4 mM significantly reduced cellular viability (Figure 15).

In both the HepG2 and HuH7 cell lines, SA at concentrations of 3.3 and 4 mM significantly reduced cellular viability at all three time-points of 2, 4 and 24 hours. There were no effects on the viability of the HepG2 and HuH7 cell lines when a concentration of 0.4 mM SA was applied at times of 2, 4 and 24 hours (Figure 16 and 17).

Hep3B

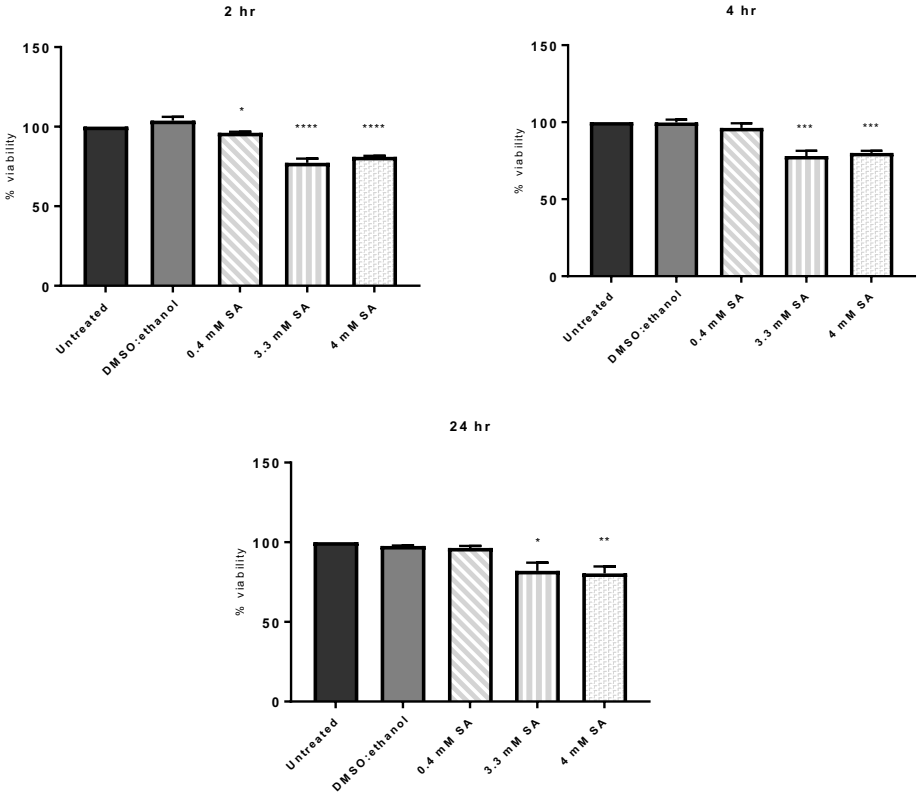


Figure 15. Effects of commercial SA on the viability of the Hep3B cell line. The resazurin assay was performed after 2, 4 and 24 hour treatments with commercial SA. **2 hr**: SA at concentrations of 0.4 mM, 3.3 mM and 4 mM all significantly reduced Hep3B viability compared to the control after 2 hour treatments (*P 0.0280, P < 0.0001*, *P < 0.0001*, respectively). **4 hr**: At concentrations of 3.3 mM and 4 mM, SA significantly reduced Hep3B viability compared to control after 4 hour treatments (*P 0.0002*, *P 0.0004*, respectively). **24 hr**: At concentrations of 3.3 mM and 4 mM, SA again significantly reduced the viability of Hep3B cells compared to the control after 24 hour treatments (*P 0.0147*, *P 0.0081*, respectively). Statistical analysis was performed using a one-way ANOVA with post-hoc Dunnett's Test vs control samples. Control sample is DMSO: Ethanol (25:75 v: v). All results are displayed as ± SEM, with statistical significance where P < 0.05. *N*=3.

HepG2

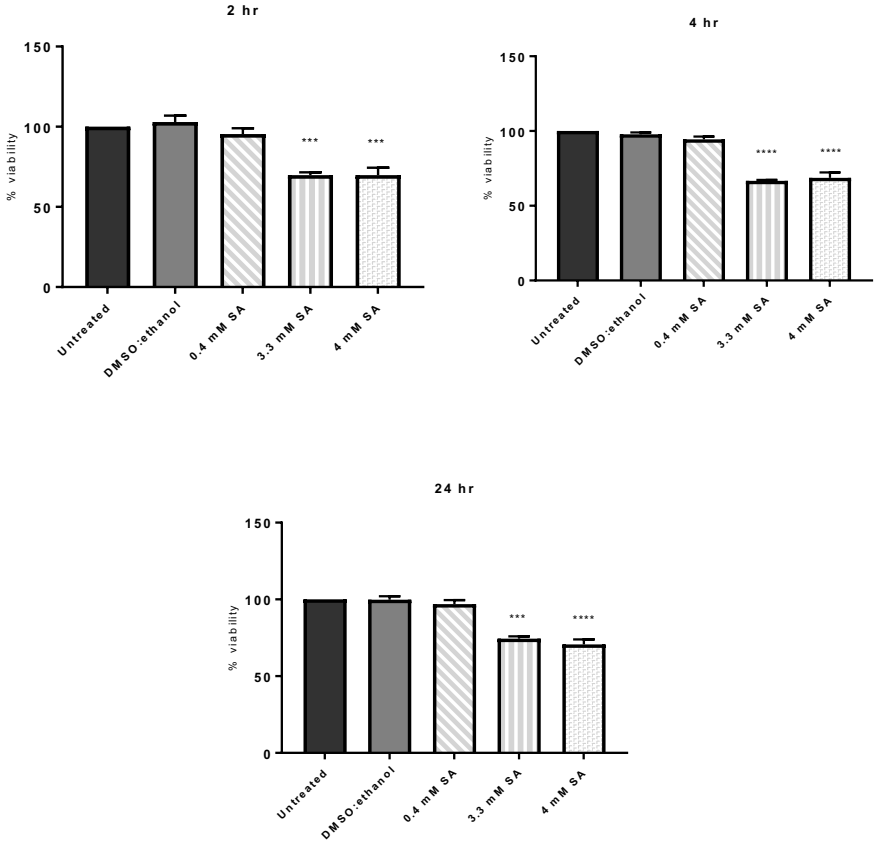


Figure 16. Effects of commercial SA on the viability of the HepG2 cell line. The resazurin assay was performed after 2, 4 and 24 hr treatments with commercial SA. **2 hr**: At concentrations of 3.3 mM and 4 mM, SA significantly reduced HepG2 viability compared to control after 2 hr treatments (*P 0.0006, P 0.0003*, respectively). **4 hr:** At concentrations of 3.3 mM and 4 mM, SA significantly reduced HepG2 viability compared to control after 4 hour treatments *(P < 0.0001* for both). **24 hr**: At concentrations of 3.3 mM and 4 mM, SA significantly reduced HepG2 viability compared to control after 24 hour treatments (*P 0.0003*, *P < 0.0001*, respectively). Statistical analysis was performed using a one-way ANOVA with post-hoc Dunnett's Test vs control samples. Control sample is DMSO: Ethanol (25:75 v: v). All results are displayed as ± SEM, with statistical significance where P < 0.05. *N*=3.

HuH7

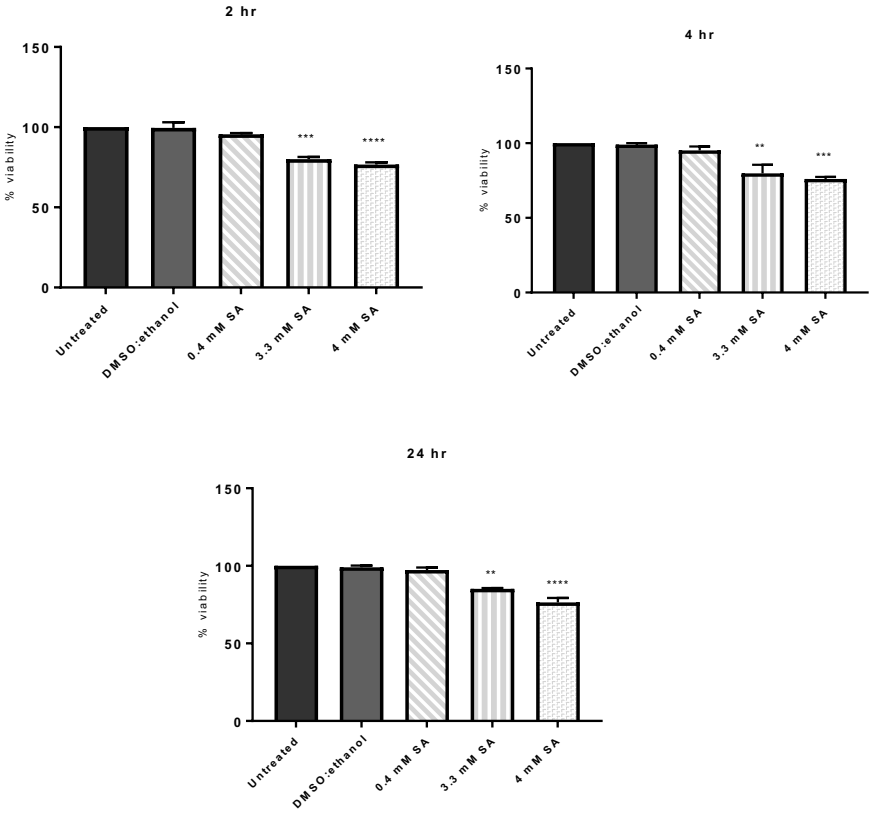


Figure 17. Effects of commercial SA on the viability of the HuH7 cell line. The resazurin assay was performed after 2, 4 and 24 hour treatments with commercial SA. **2 hr**: At concentrations of 3.3 mM and 4 mM, SA significantly reduced HuH7 viability compared to control after 2 hr treatments (*P 0.0003, P <0.0001*, respectively). **4 hr:** At concentrations of 3.3 mM and 4 mM, SA significantly reduced HuH7 viability compared to control after 4 hour treatments (*P 0.0011, P 0.0001*, respectively). **24 hr**: At concentrations of 3.3 mM and 4 mM, SA significantly reduced HuH7 viability compared to the control after 24 hour treatments (*P 0.0017, P <0.0001*, respectively). Statistical analysis was performed using a one-way ANOVA with post-hoc Dunnett's Test vs control samples. Control sample is DMSO: Ethanol (25:75 v: v). All results are displayed as ± SEM, with statistical significance where $P < 0.05$. *N*=3.

4.3.2.2 Effects of phenolic extract II on the viability of liver and human intestinal cell lines

The resazurin assay was performed to determine the effects of extract II on cellular viability, using the cell lines HuH7, HepG2, Hep3B and Caco-2. The liver cell lines were chosen as they are commonly used in toxicity screens, and also in studies relating to diabetes (Sefried *et al.*, 2018; González *et al.*, 2017). The Caco-2 cell line was used as it serves as a model of the intestinal barrier, which is the main area of metabolism if the extract was to be potentially utilised as a functional food ingredient.

Extract II at a concentration of 1 mg/mL significantly affected the viability of Caco-2 cells after a 4 hour treatment. There were no significant effects observed at 2 or 24 hours for concentrations of extract II less than 1 mg/mL (Figure 18).

Extract II significantly reduced viability of the Hep3B cell line at 1 and 0.75 mg/mL after 24 hour treatments (Figure 19). Viability of the HepG2 cell line was significantly reduced at 1 and 0.75 mg/mL after 2 hour treatments, while only 1 mg/mL significantly reduced viability after 4 and 24 hour treatments (Figure 20). Finally, a concentration of 1 mg/mL of extract II significantly reduced the viability of the HuH7 cell line at all three time-points (Figure 21).

Caco-2

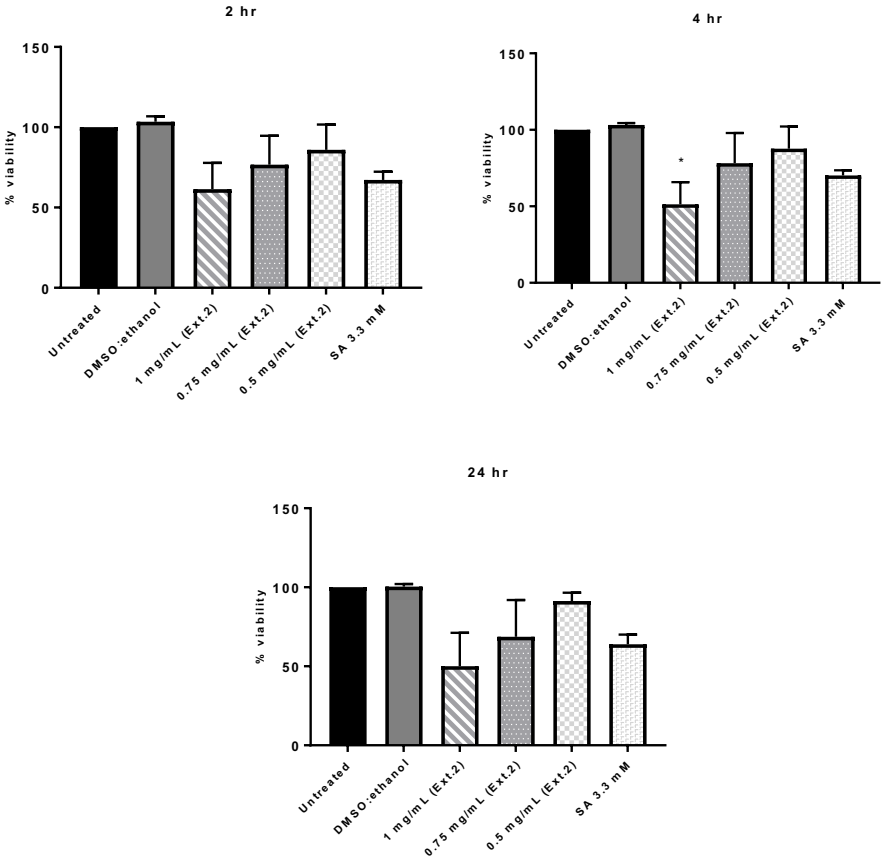


Figure 18. Effects of commercial SA and phenolic extract II on the viability of the Caco-2 cell line. The resazurin assay was performed after 2, 4 and 24 hour treatments with phenolic extract II and commercial SA. **2 hr**: Extract II and commercial SA assayed at concentrations ranging from 1-0.5 mg/mL, did not affect cellular viability compared to the control. **4 hr:** At a concentration of 1 mg/mL, extract II significantly reduced Caco-2 viability compared to the control (*P 0.0332*). Commercial SA did not affect cellular viability. **24 hr**: None of the concentrations tested for extract II and commercial SA affected cellular viability compared to the control. Statistical analysis was performed using a one-way ANOVA with a post-hoc Dunnett's Test vs control samples. Control sample is DMSO: Ethanol (25:75 v: v). All results are displayed as ± SEM, with statistical significance where $P < 0.05$. *N*=3.

Hep3B

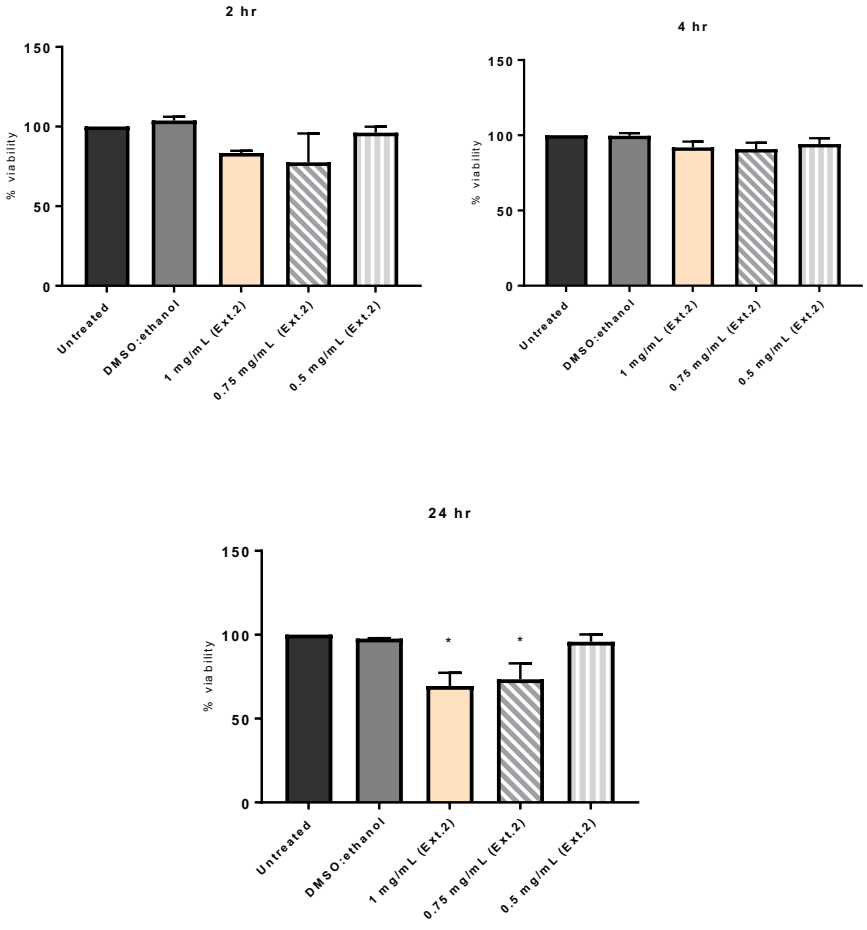


Figure 19. Effects of phenolic extract II on the viability of the Hep3B cell line. The resazurin assay was performed after 2, 4 and 24 hour treatments with phenolic extract II. **2 hr**: Extract II did not affect cellular viability when compared to the control after 2 hr treatments. **4 hr:** Extract II did not affect cellular viability. **24 hr**: At concentrations of 1 and 0.5 mg/mL, extract II significantly reduced Hep3B viability (P 0.0191 and P 0.0223, respectively). Statistical analysis was performed using a one way ANOVA with a post-hoc Dunnett's Test vs control samples. Control sample is DMSO: Ethanol (25:75 v: v). All results are displayed as ± SEM, with statistical significance where $P < 0.05$. *N*=3.

HepG2

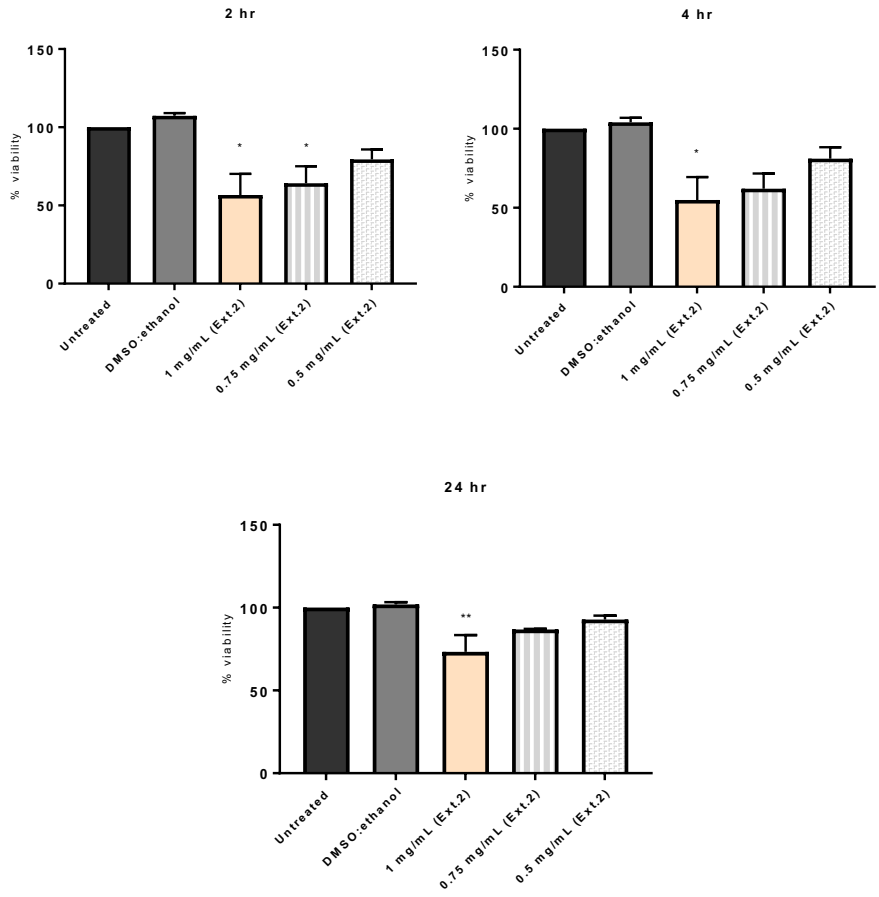


Figure 20. **Effects of phenolic extract II on the viability of the HepG2 cell line**. The resazurin assay was performed after 2, 4 and 24 hour treatments with phenolic extract II. **2 hr**: Extract II at concentrations of 1 and 0.75 mg/mL respectively, significantly reduced cellular viability of HepG2 cells when compared to the control (*P 0.0221, 0.0412*, respectively). **4 hr:** Extract II at a concentration of 1 mg/mL significantly reduced cellular viability of HepG2 cells when compared to the control (*P0.0277*). **24 hr**: Extract II at 1 mg/mL significantly reduced cellular viability when compared to the control (*P 0.0029*). Statistical analysis was performed using a One-way ANOVA with a post-hoc Dunnett's Test vs control samples. Control sample is DMSO: Ethanol (25:75 v: v). All results are displayed as ± SEM, with statistical significance where $P < 0.05$. *N*=3.

HuH7

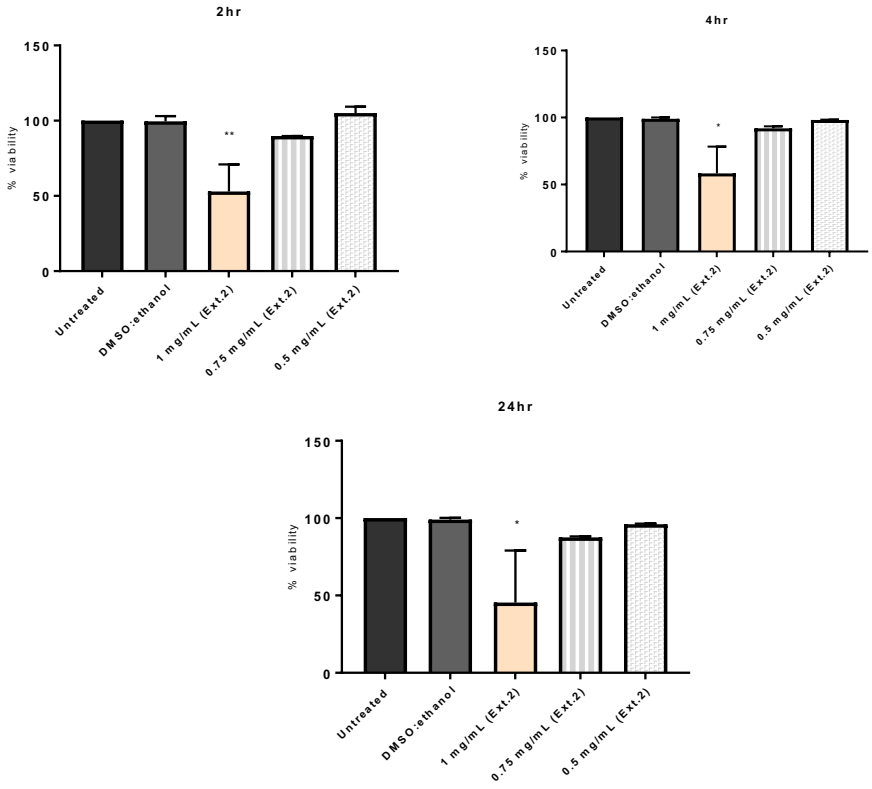


Figure 21. **Effects of phenolic extract II on the viability of the HuH7 cell line**. The resazurin assay was performed after 2, 4 and 24 hour treatments with phenolic extract II. **2 hr**: Extract II at a concentration of 1 mg/mL significantly reduced cellular viability when compared to the control (*P 0.0054*). **4 hr:** Extract II at a concentration of 1 mg/mL significantly reduced cellular viability when compared to the control (*P 0.0135*) **24 hr**: Extract II at 1 mg/mL significantly reduced cellular viability when compared to control (*P 0.0417*). Statistical analysis was performed using a one-way ANOVA with a post-hoc Dunnett's Test vs control samples. Control sample is DMSO: Ethanol (25:75 v: v). All results are displayed as ± SEM, with statistical significance where $P < 0.05$. *N*=3.

4.3.3 Summary of cell line toxicity data for phenolic extracts and commercial SA

Below is a summary of the various cell lines used, along with the corresponding concentrations of phenolic extracts I, II and commercial SA which were toxic and non-toxic in selected cell lines (expressed in mg/mL).

Cell line	Extract I		Extract II		Commercial SA	
	Toxic	Non-toxic	Toxic	Non-toxic	Toxic	Non-toxic
H9c2	1	0.75	-	-	1 mM	-
HuH7	1	0.5	1	0.75	4 mM	0.4 mM
SGBS	-	1	-	-	-	1 mM
Hep3B	-	-	1	0.5	4 mM	0.4 mM
HepG2	-	-	1	0.5	4 mM	0.4 mM
Caco-2	-	-	1	0.5	3.3 mM	-

Table 7. Toxic and non-toxic concentrations of phenolic extracts I and II (mg/mL), along with commercial SA (mM) in various cell lines

4.3.4 Phenolic extract I and commercial SA do not effect GLUT4 expression after 48 hour treatments

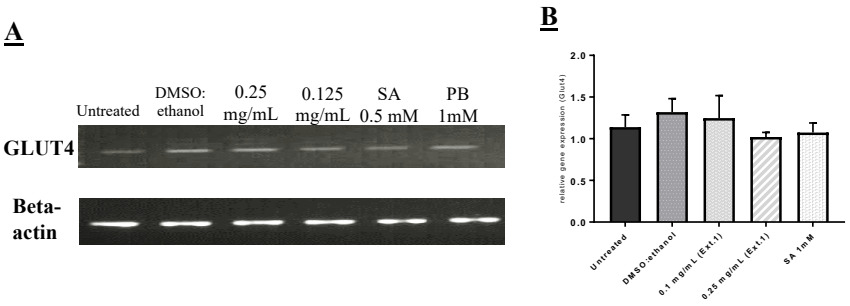
Previous work by O'Byrne *et al.* (2011) has shown that GLUT4, which is the main insulin-responsive glucose transporter, is regulated by HDACs. Given SA has previously been shown to inhibit HDAC activity, the effect of the phenolic extracts and commercial SA on GLUT4 expression were examined.

Phenolic extract I was used at a maximum concentration of 0.25 mg/mL dissolved in DMSO: Ethanol (25:75 v: v), as this was the dose at which there were no effects on cellular viability observed in both the HuH7 and H9c2 cell lines (Figure 22). After 48 hour independent-treatments with phenolic extract I and commercial SA, RNA was collected and reverse transcribed to cDNA. GLUT4 gene expression was determined by end-point PCR with agarose gel electrophoresis and quantitative PCR (Figure 22).

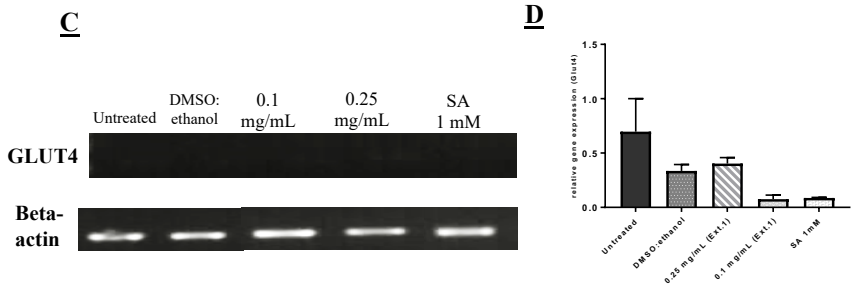
Both analyses showed there were no effects on GLUT4 expression in the HuH7, H9c2 or SGBS cell lines following treatments with extract I and commercial SA.

-End-point PCR- *-qPCR analysis-*

H9c2



HuH7



SGBS

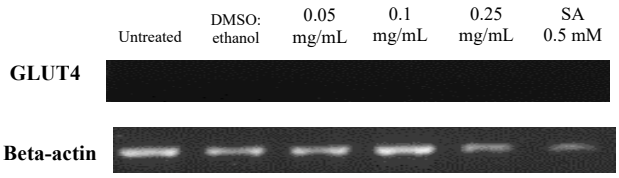


Figure 22. GLUT4 expression in H9c2, HuH7 and SGBS cell lines after 48 hour treatments. ***H9c2 cell line:*** There are no effects on GLUT4 expression in the H9c2 cell line after 48 hour treatments with extract I using (**A**) end-point PCR and (**B**) using qPCR analysis. ***HuH7 cell line***: There is no significant difference in GLUT4 expression in the HuH7 cell line after 48 hour treatments with extract 1 using (**C**) end-point PCR and (**D**) using qPCR analysis. ***SGBS cell line***: There was no difference in GLUT4 expression observed after 48 hour treatments with extract 1 and commercial SA using end-point PCR. Statistical analysis was performed using a one-way ANOVA with a post-hoc Dunnetts test (vs control, $p < 0.05$). *N*=3

4.3.5 GLUT4 expression in the H9c2 cell line is not effected after 24 hour treatments with extract I, II and commercial SA

As there were no effects on GLUT4 expression after 48 hour treatments, it was decided to re-analyse the effects of extract I and commercial SA in a 24 hour treatment experiment, along with extract II.

Both phenolic extracts I and II at a concentration of 0.5 mg/mL did not significantly effect GLUT4 expression after 24 hour treatments in the H9c2 cell line. Commercial SA also did not affect GLUT4 expression, even at the increased concentration of 2.2 mM for 24 hours. (Figure 23).

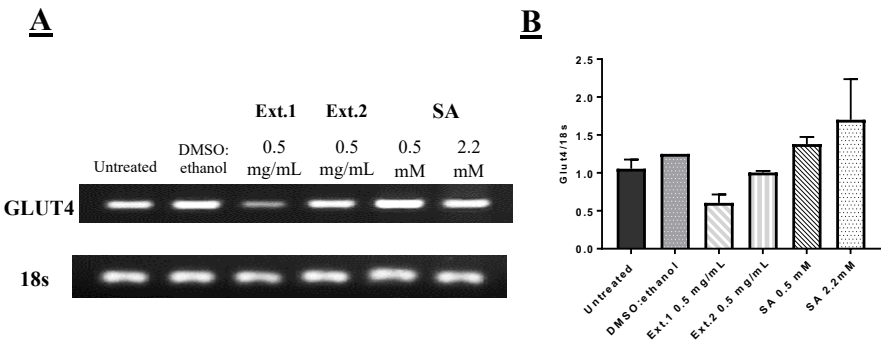


Figure 23. **GLUT4 expression in the H9c2 cell line after 24 hour treatments.** Both phenolic extracts I and II at 0.5 mg/mL, and commercial SA at 0.5 and 2.2 mM do not significantly affect GLUT4 expression when analysed by (A) end-point PCR and (B) densitometric analysis. Statistical analysis was performed using a one-way ANOVA with a post-hoc Dunnetts test (vs control, $p < 0.05$). *N*=3.

4.3.6 Commercial SA increases glucose uptake, while phenolic extract I inhibits uptake in muscle cells

The effect of both phenolic extracts I and II along with commercial SA, was determined in terms of ability to influence glucose uptake in H9c2 cells. A concentration of 1 mg/mL displayed cellular toxicity and so a concentration of 0.5 mg/mL was chosen for each extract, while 2.2 mM was chosen for SA. (These were also the same concentrations used to determine their effects on GLUT4 expression).

The cells were incubated independently for 2 hours with the extracts and SA, followed by a further 1 hour incubation with Phloretin, a known glucose uptake inhibitor. Extract II at a concentration of 0.5 mg/mL significantly inhibited glucose uptake compared to the DMSO: Ethanol (25:75 v: v) control (grey), while extract I at the same concentration does not seem to effect glucose uptake (Figure 24). Commercial SA at a concentration of 2.2 mM significantly increased glucose uptake compared to the control (grey). ($P < 0.05$) (Figure 24).

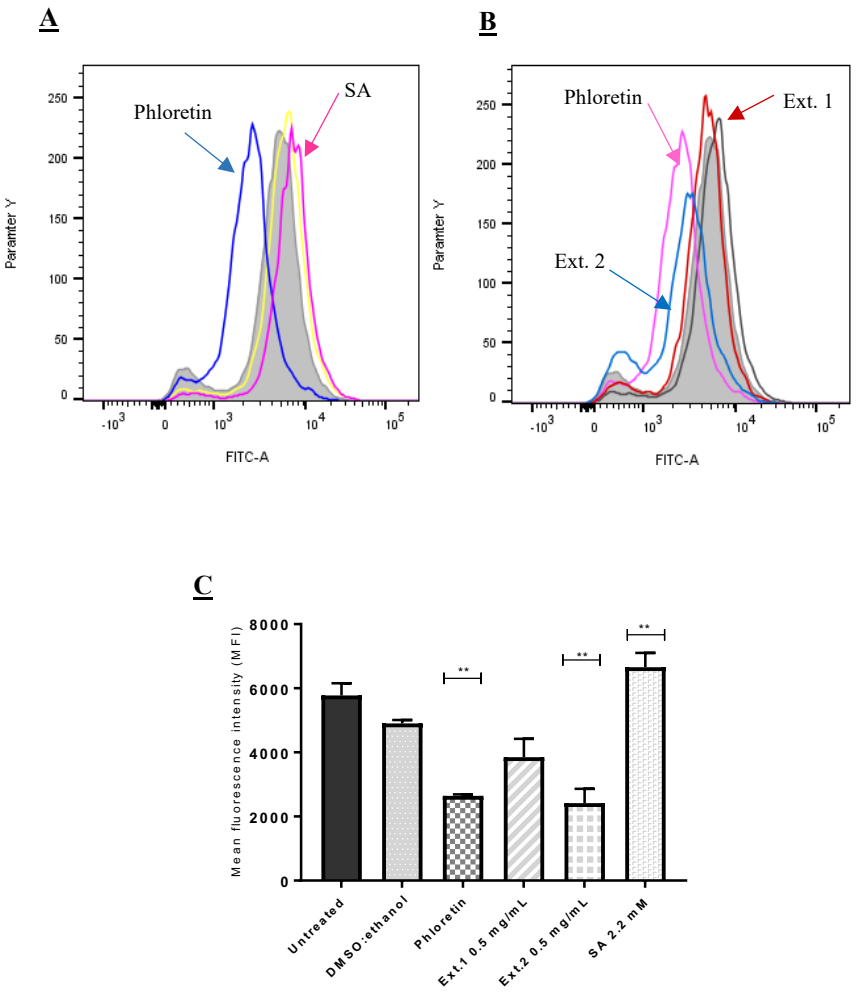


Figure 24. **Effects of extracts, I, II and commercial SA on Glucose uptake in H9c2 cells**. **(A)** Compared to the control (grey) sample, commercial SA at a concentration of 2.2 mM increases glucose uptake. **(B)** Compared to the control (grey) sample, extract 1 at 0.5 mg/mL does not seem to influence glucose uptake. Extract II at the same concentration inhibits glucose uptake similar to that of Phloretin, which is a known glucose uptake inhibitor. (**C**) Extract II significantly inhibits glucose uptake, while SA significantly increases glucose uptake after 2 hour treatments. Statistical analysis was performed using a one-way ANOVA with a post-hoc Dunnetts test (vs control; $p < 0.05$). *N*=3.

4.4 Discussion

The first aim of this chapter was to examine the histone deacetylase inhibitory activity of commercial SA, given only one study has shown it acts as a HDAC inhibitor. Histone deacetylase's are enzymes responsible for removing acetyl groups from lysine residues on histone tails (Yoon and Eom, 2016). As a result, they play an important role in regulating gene expression and protein modification. HDAC inhibitors have been shown to play a role in various diseases such as Cardiovascular, Cancer, Inflammatory Cystic-Fibrosis Lung Disease, Parkinson's disease, and Diabetes (Yoon and Eom, 2016; Bodas *et al.*, 2018; Tan *et al.*, 2018; Besancon *et al.*, 2018; Cho *et al.*, 2018). Using a HDACi assay kit, commercial SA was tested at various concentrations, along with TSA, which is a potent non-selective HDAC inhibitor. Significant inhibition was observed with the HDAC assay at the highest concentrations of SA, 10 and 20 mM, after 24 hour treatments. There was no effect after 3 hours with commercial SA, however there was with the positive control TSA. It was aimed to confirm this by Western Blot analysis, at the time-points of 3 and 24 hours. After 3 hour treatments with SA, there was no HDAC inhibitory activity observed. However, at a concentration of 10 mM, the band density appears to be increasing which supports the hypothesis that SA acts as a HDAC inhibitor only at high concentrations. At a concentration of 2.2 mM, SA resulted in a decrease in band density. In contrast, the positive control SAHA shows a clear increase in band density,

as acetylation is increased due to its role as a HDACi. After 24 hours, SA at concentrations of 2.2 and 4.46 mM caused a decrease in band density, indicating there is less histone H3 acetylation occurring. One possible explanation for the difference observed between 3 and 24 hours in the HDAC assay could be due to the presence of metabolites which are functioning as HDAC inhibitors. Sinapinic acid is known to be metabolised to 3-hydroxy-5-methoxyphenylpropionic acid, dihydrosinapic acid and 3-hydroxy-5-methoxycinnamic acid (Chen, 2016). In terms of its structure, dihydrosinapic acid is only lacking in one double bond in comparison to SA, and so should behave similarly. As such, 24 hours may be enough time for SA to be metabolised and it could be these metabolites which are contributing to the observed HDAC inhibition seen at 24 hours in the HDAC assay. Unfortunately, we could not confirm that SA at a concentration of 10 mM acts as a HDAC inhibitor at 24 hours, as insufficient amounts of histone proteins could be extracted. This could be due to the degradation of histone proteins within the cells due to the toxic concentration of SA applied. It appears from the data that that SA acts in a dose-dependent manner, with band density increasing as the concentration increases. Zinc is crucial for the catalytic activity of HDAC enzymes, and it has been shown that phenolic acids inhibit the ACE-I enzyme by binding zinc and blocking the catalytic activity of the ACE-I enzyme. Indeed, data from chapter 3 has shown that commercial SA can inhibit the ACE-I enzyme (Goncalves and Romano, 2017).

However, this inhibition was observed when SA was used at concentrations of 5 mM. Therefore it appears that SA is only effective in the H9c2 cell line at concentrations which reduce cell viability. Other studies have examined the effects of phenolic acids, including SA, on histone H3 acetylation. Similar to the results obtained here, they found that SA increased histone H3 acetylation in a dose-dependent manner, and to different extents dependent on the cell line. In the MCF-7 breast cancer cell line, SA at concentrations of 3 and 4 mM produced very faint bands on a Western Blot, while a large band appears when concentrations of 5 mM are applied to MCF-7 cells, indicating the SA acts as a HDACi at the highest concentration (Saenglee *et al.*, 2016). In the HeLa cervical cancer cell line, Western Blot analysis of SA produced small bands when concentrations of 1 and 2 mM were applied, with a large band appearing when a concentration of 3 mM was applied (Saenglee *et al.*, 2016). This demonstrates that the role of SA as a HDACi is dose and cell line dependent, and so further study is necessary using different cell lines and a broader range of concentrations. The data also suggests that SA could prove useful in a cancer setting, as previous studies have also found that SA is a cytotoxic agent against various cancer cell lines, and induces apoptosis at high concentrations (Eroğlu *et al.*, 2017; Saenglee *et al.*, 2016).

There is scant evidence in the current literature regarding the toxicity of commercial SA, and what does exist shows variation in the level

of toxicity observed. A study by Saenglee *et al*. (2016) found that commercial SA had different IC_{50} values in two different cancer cell lines. For MCF-7 breast cancer cells, the IC_{50} was greater than 5 mM, while for HeLa cervical cancer cells the IC_{50} was 2.6 mM, after 24 hours of treatment. A 'normal' cell line, Vero kidney epithelial cells were also found to have an IC_{50} greater than 5 mM (Saenglee *et al.*, 2016). In contrast, another study by Hameed *et al*. (2016) found that SA significantly reduced HeLa cell viability starting from 5 mM, with an IC_{50} of 7.24 mM after an 18 hour exposure. The group also tested V79 Chinese hamster lung fibroblasts for 18 hours with SA, and were found to have an IC_{50} of 1.86 mM. These studies have shown that SA has a different toxicity profile depending on cell type, treatment time and the concentration of SA used. It was important to assess the extracts and commercial SA to determine a non-toxic dose in cell lines relevant to the experiments performed in this work.

The main aim of this chapter was to investigate any potential anti-diabetic effects of the extracts I, II and commercial SA. Muscle tissue is the main site of insulin-stimulated glucose uptake *in-vivo*, and so the H9c2 muscle cell line was chosen (Michael *et al.*, 2001). Many studies have also shown that the effect of insulin on adipose tissues is dependent on GLUT4, and the SGBS cell line was also used as part of the cytotoxicity studies (Hauner *et al.*, 1998). Three liver cell lines were also used as part of the toxicity studies with the extracts and SA. Previous work by O'Byrne et al. (2011) showed that GLUT4 is epigenetically regulated in the HepG2 cell line.

Therefore, we tested the liver cell lines HepG2, Hep3B and HuH7. Despite its tumorigenic origin, the HepG2 cell line was found to best represent the *in-vivo* setting of the liver (Sefried *et al.*, 2018). The HuH7 liver cell line is also relevant in that it has been used to study iron metabolism in obesity, with obesity known to play a key role in the pathology of T2DM (Chung *et al.*, 2007). The Caco-2 cell line was also tested as SA is primarily absorbed in the small intestine, where Caco-2 cells serve as a model of the human intestinal barrier.

The initial toxicity studies were carried out with extract I and commercial SA for 48 hours. Since extract II was generated after extract I, it was subsequently tested along with commercial SA for incubation times up to 24 hours in liver cell lines. As seen in table 7, both extracts at concentrations of 1 mg/mL were toxic. A concentration of 0.5 mg/mL of extracts I and II was non-toxic in the tested cell lines. In terms of commercial SA, higher concentrations of 4 mM reduced cell viability, with only concentrations of 0.4 mM having no effect on cell viability in the cell lines tested.

GLUT4 is a key regulator of glucose uptake in response to insulin, and its dysregulation plays a key role in the development of T2DM. Initial experiments were carried out with extract I to determine if it effects GLUT4 expression in the SGBS pre-adipocyte cell line and HuH7 liver cell line after 48 hour treatments. There was no GLUT4 detected in either cell line, and treatments with either extract I or commercial SA do not increase its expression. Previous work has

shown that there is very little GLUT4 expression in pre-adipocytes, with an increase in GLUT4 corresponding to a concomitant increase in differentiation. It may therefore be necessary to differentiate the SGBS cell line and then examine the expression of GLUT4 after treatments with extract I and commercial SA (Hauner *et al.*, 1998). Extract I and commercial SA were also assessed for their effects on GLUT4 expression in the H9c2 muscle cell line. There was no effect on GLUT4 expression observed after a 48 hour treatment in H9c2 cells. However following treatment with extract I and commercial SA after 24 hours, there was greater expression of GLUT4, even in untreated samples. Extract II was also tested with extract I and commercial SA, both at higher concentrations and for 24 hours. There was an increase in GLUT4 expression with commercial SA at concentrations of 0.5 and 2.2 mM. However extract I at a concentration of 0.5 mg/mL caused a decrease in GLUT4 expression, while extract II at a concentration of 0.5 mg/mL also decreases GLUT4 expression. GLUT4 expression increased after 24 hours in the untreated and control cells, it may be necessary to repeat this experiment in differentiated SGBS cells and the HuH7 cell line after 24 hour treatments and at higher concentrations.

Along with assessing GLUT4 expression, the ability of both extracts and commercial SA to influence glucose uptake was assessed. Given muscle is the main site of insulin-stimulated glucose uptake, the H9c2 cell line was used. Commercial SA at a concentration of 2.2 mM was found to significantly increase glucose uptake in H9c2

muscle cells when compared to control cells. In contrast, both extracts resulted in a decrease in glucose uptake, with extract II at a concentration of 0.5 mg/mL resulting in a significant decrease in uptake. This is reflected in GLUT4 expression, where the extracts displayed a trend towards decreased GLUT4 expression. However, the result obtained with commercial SA correlates with a previous study by Cherng *et al*. (2013), who demonstrated that SA increased glucose uptake in the soleus muscle of diabetic rats. This increase in uptake was observed after a 2 hour pre-treatment with commercial SA, and therefore it might be of benefit to assess GLUT4 expression at this time-point also. Interestingly, the same study by Cherng *et al*. (2013) found that *in-vivo*, it took repeated treatments with SA before an increase in GLUT4 gene expression was observed. One 24- hour treatment may not have been long enough to effect GLUT4 expression, and perhaps repeated treatments at shorter time-points might prove effective.

Obesity is known to increase the risk of developing T2DM and insulin resistance. Adipocytes can also secrete pro-inflammatory cytokines, and SA was shown to possess anti-inflammatory activity as shown in Chapter 5. Therefore SA could still prove effective in managing some of the underlying pathological features of diabetes.

Although there have been some negative results with both the extracts and commercial SA, it still has potential as a therapeutic agent for diabetes. *In-vivo* studies are crucial to determining the

benefits of these extracts, as studies have shown that commercial SA is well tolerated in animal models (Cherng *et al.*, 2013). It is therefore crucial to determine a non-toxic dose of the phenolic extracts which are efficacious. Indeed, a concentration of each extract which is high enough to have an effect could be tolerated *in-vivo*. Animal models are also superior to individual cell lines, especially when studying complex diseases such as diabetes. Significantly, *in-vivo* studies have shown that commercial SA does have a role in the treatment and prevention of diabetes, and though further studies are necessary, the phenolic extracts could still have potential for use as therapeutic agents in diabetes prevention and treatment (Kanchana *et al.*, 2011; Cherng *et al.*, 2013).

Chapter 5

Assessment of biological activities II:

Inflammation

5.1 Introduction

Inflammation is the immune systems response to harmful stimuli including pathogens, damaged cells or toxic compounds (Chen *et al.*, 2018). Inflammation is a defence mechanism necessary for health, however if the checks and balances of inflammation break down chronic inflammation can occur which has serious consequences for health. Chronic inflammation plays a key role in the underlying pathology of numerous diseases including cardiovascular disease, arthritis, diabetes (T2DM) and bowel disease (Chen *et al.*, 2018). The inflammatory process begins with recognition of harmful stimuli by Pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs), which upon stimulation activate nuclear translocation of transcription factors including NF-κB (Chen *et al.*, 2018). Lipopolysaccharide (LPS) is a constituent in the outer wall of gram-negative bacteria, and is recognised by TLR4. The activation of TLR4 by LPS in turn stimulates the activation of NF-κB, which results in the production of pro-inflammatory cytokines (Yang *et al.*, 2016). Monocytes are one of the first group of cells to be recruited to the site of infection, and differentiate into macrophages and dendritic cells. Macrophages are a key component of the inflammatory process, from initiation to resolution. They also produce a wide range of cytokines which modulate the immune response (Chen *et al.*, 2018). Cytokines can either be pro- or anti-inflammatory, which either aide or inhibit inflammation, respectively. The primary function of cytokines is to recruit

leukocytes to the site of infection or injury, however excessive production of pro-inflammatory cytokines can lead to tissue damage, organ failure and ultimately can prove fatal (Chen *et al.*, 2018). Pro-inflammatory cytokines are implicated in the pathology of diseases including arthritis and inflammatory bowel diseases. The most common form of arthritis is osteoarthritis (OA), with the knee joint most commonly affected. Obesity is a key risk factor in the development of OA, with inflammatory cytokines the link between both conditions. Macrophages derived from adipose tissue secrete TNF-alpha, IL-6 and Il-1, which in turn play a role in cartilage matrix degradation and bone resorption in OA. In patients with OA, the levels of these inflammatory cytokines were found to be elevated in synovial fluid, synovial membrane and sub-chondral bone and cartilage (Wang and He, 2018). The same cytokines, TNF-alpha, IL-1β and IL-6, were also found to be increased in patients with Inflammatory Bowel Disease (IBD) compared to healthy controls (Singh *et al.*, 2016). As such, blocking these cytokines is a mechanism to treat inflammation and its associated diseases.

Cytokine	***Source***	***Function***
TNF-alpha	• Macrophages • Adipocyte • NK cells • $CD4^+$ lymphocytes	▪ Pro-inflammatory ▪ Cytokine production ▪ Cell proliferation ▪ Apoptosis
IL-1β	• Macrophages • Monocytes	▪ Pro-inflammatory ▪ Proliferation ▪ Apoptosis
IL-8	• Macrophages • Epithelial cells • Endothelial cells	▪ Pro-inflammatory ▪ Chemotaxis ▪ Angiogenesis
IL-6	• Macrophages • T-cells • Adipocyte	▪ Pro-inflammatory ▪ Differentiation ▪ Cytokine production
IL-12	• Dendritic cells • Macrophages • Neutrophils	▪ Pro-inflammatory ▪ Cell differentiation ▪ NK cell activation

Table 8. A list of pro-inflammatory cytokines, along with their source and biological functions.

Current treatments for conditions where inflammation is the underlying factor, include non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs are one of the most commonly prescribed medications, accounting for approximately 5-10 % of all prescriptions each year (Wongrakpanich *et al.*, 2018). However, NSAIDs have adverse effects, particularly on the gastrointestinal system. Serious effects include intestinal bleeding, peptic ulcers and GI perforation. Research is moving toward natural products as an alternative, which possess anti-inflammatory activity with minimal side effects on the GI system (Ambriz-Pérez *et al.*, 2016). In line with this move toward natural anti-inflammatory agents, this chapter focused on assessing the anti-inflammatory effects of extract I and II generated from Irish rapeseed meal, along with commercial SA.

5.2 Aims and objectives

The main aim of this chapter was to examine the anti-inflammatory properties of extract I and II generated from Irish rapeseed meal, along with commercial SA.

Specific objectives:

1. To isolate human PBMC's and determine the effects of both extracts and commercial SA on PBMC viability.
2. To perform ELISA assays to determine the anti-inflammatory effects of both extracts and commercial SA in human PBMCs.

5.3 Results

5.3.1 Determining the effect of commercial SA on the viability of human peripheral blood mononuclear cells (PBMCs)

In order to study the effects of commercial SA on inflammation, we used the model of LPS-stimulated peripheral blood mononuclear cells (PBMCs). It was first necessary to determine that concentrations of commercial SA which were non-toxic to the isolated PBMC's.

Commercial SA was added to the isolated PBMC's at various concentrations for 3 hours, followed by the addition of LPS (20 ng/mL) for 2, 4 and 6 hours. After each time-point, resazurin solution was added to each well and measured spectrophotometrically at 595 nM.

After 2 hours, commercial SA at concentrations of 2.5 and 10 mM significantly reduced PBMC viability (P 0.0044; P <0.0001, respectively). After 6 hours, commercial SA caused a significant reduction in viability at 10 mM (P 0.0028) (Figure 25).

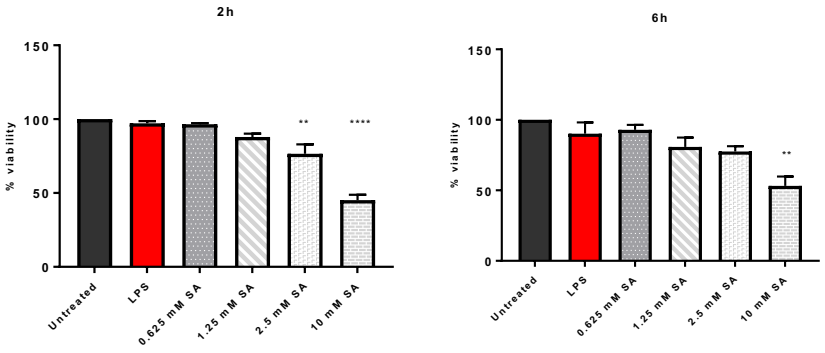


Figure 25. Viability of human PBMCs after treatments with commercial SA. Isolated PBMC's were treated with SA for 3 hours, after which LPS (20 ng/mL) was added for 2 and 6 hours. The resazurin assay was then used to determine percent viability. **2 hr:** At concentrations of 2.5 and 10 mM, cellular viability was significantly reduced (P 0.0044; P <0.0001, respectively). **6 hr**: At a concentration of 10 mM, SA significantly reduced viability (P 0.0028). Statistical analysis was performed using a Dunnett's Test vs LPS samples. Results are displayed as ± SEM, with statistical significance where $P < 0.05$. (N=3).

5.3.2 Commercial SA significantly reduces LPS-induced TNF-alpha expression in human PBMCS

TNF-alpha is known to play a key role in inflammation and inflammatory diseases. In order to determine if commercial SA possess anti-inflammatory activity, isolated PBMCs were pre-treated with commercial SA for 3 hours followed by the addition of LPS (20 ng/mL) for 2, 4 and 6 hours.

After 2 hours, SA at concentrations of 1.25, 2.5 and 10 mM all significantly reduced LPS-induced TNF-alpha expression (P 0.0327; P 0.0046; P 0.0002, respectively). After 4 hours, SA also significantly reduced TNF-alpha at all concentrations tested: 1.25, 2.5 and 10 mM (P 0.0033; P 0.0004; P <0.0001, respectively) (Figure 26). Finally, after 6 hours, SA significantly reduced TNF-alpha expression at 2.5 and 10 mM (P 0.0320 and P 0.0142, respectively) (Figure 26).

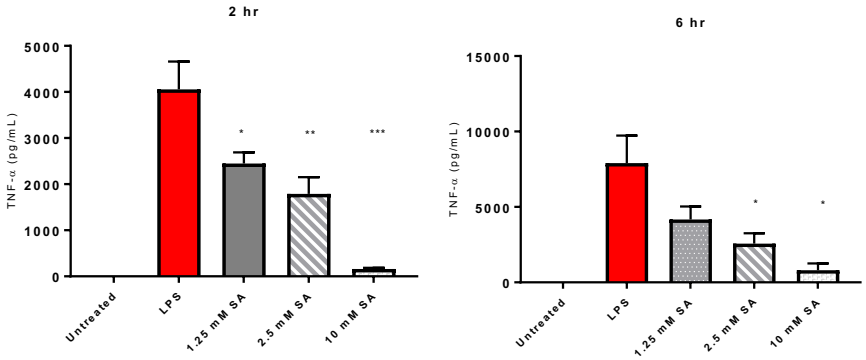


Figure 26. **Effect of commercial SA on LPS-induced TNF-alpha expression in human PBMC's.** Isolated PBMC's were treated with commercial SA for 3 hours, followed by 2,4 and 6 hour incubations with LPS (20 ng/mL). **2h**: Commercial SA significantly reduces TNF-alpha at a non-toxic concentration of 1.25 mM (P 0.0327). **6h:** Commercial SA significantly reduces TNF-alpha expression at a non-toxic concentration of 2.5 mM (P 0.0320). Statistical analysis was performed using a Dunnett's Test vs LPS samples. Results are displayed as ± SEM, with statistical significance where $P < 0.05$. (N=3).

5.3.3 Commercial SA significantly reduces LPS-induced Il-1β expression in human PBMC's

IL-1β has been implicated in a growing number of autoinflammatory diseases and reduction in IL-1β has been shown to be of benefit in diseases such as type II diabetes and heart failure (Dinarello, 2011).

Isolated PBMCs were pre-treated with commercial SA for 3 hours followed by the addition of LPS (20 ng/mL) for 2, 4 and 6 hours.

After 2 hours, SA significantly reduced IL-1β expression at 1.25, 2.5 and 10 mM ($P < 0.0001$). After 6 hours, IL-1β expression was significantly reduced at 2.5 and 10 mM SA (P 0.0449; P 0.0003, respectively) (Figure 27).

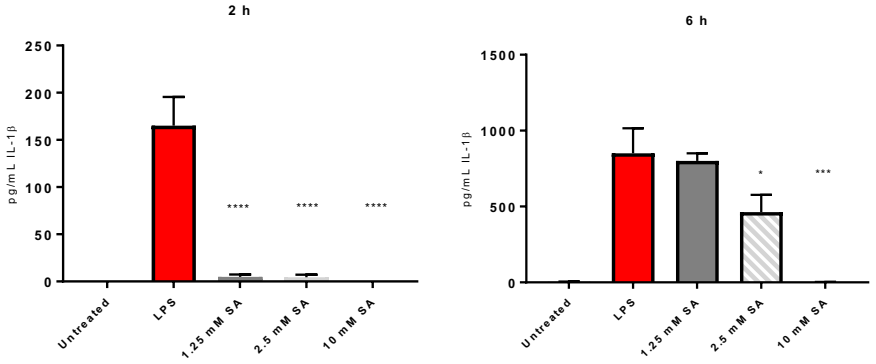


Figure 27. **Effect of commercial SA on LPS-induced IL-1β expression in human PBMC's.** Isolated PBMC's were treated with commercial SA for 3 hours, followed by 2,4 and 6 hour incubations with LPS (20 ng/mL). **2h**: SA significantly reduced IL-1β expression at a non-toxic concentration of 1.25 mM ($P < 0.0001$). **6h:** At a non-toxic concentration of 2.5 mM, commercial SA significantly reduced IL-1β (P 0.0449). Statistical analysis was performed using a Dunnett's Test vs LPS samples. Results are displayed as ± SEM, with statistical significance where $P < 0.05$. (*N*=3).

5.3.4 Commercial SA significantly inhibits LPS-induced IL-12 expression in human PBMC's

IL-12 is a pro-inflammatory cytokine secreted by macrophages, monocytes, dendritic cells, granulocytes and B-cells in response to pathogens. It is thought to play a role in various diseases including: multiple sclerosis, inflammatory bowel diseases and cancer (Zundler and Neurath, 2015).

Commercial SA did not have any significant effects on IL-12 expression after 2 hours (Data not shown). After 4 hours, SA significantly reduced IL-12 expression at concentrations of 2.5 and 10 mM (P 0.0291; P 0.0131, respectively). Following 6 hours with LPS, SA significantly reduced IL-12 expression at 1.25, 2.5 and 10 mM (P 0.0410; P 0.0028; P 0.0014, respectively) (Figure 28).

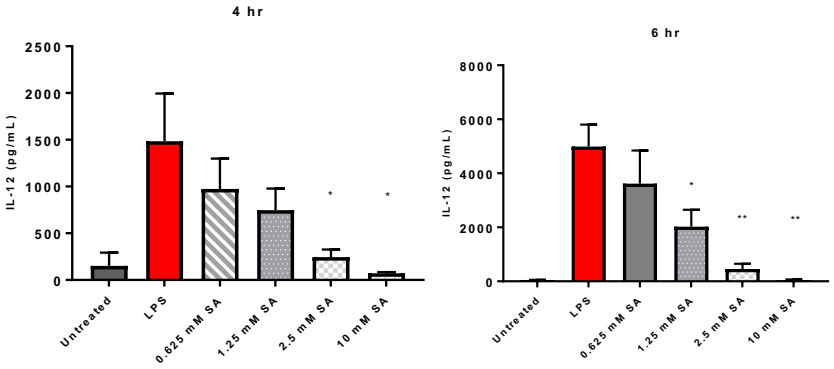


Figure 28. Effects of commercial SA on LPS-induced IL-12 expression in human PBMCs. Isolated PBMC's were treated with commercial SA for 3 hours, followed by 2,4 and 6 hour incubations with LPS (20 ng/mL). **4 h**: SA significantly reduced IL-12 expression at 2.5 and 10 mM, however at these concentrations cell viability is reduced. **6 h**: SA significantly reduced IL-12 expression at non-toxic concentrations of 1.25 and 2.5 mM (P 0.0410; P 0.0028). Statistical analysis was performed using a Dunnett's Test vs LPS samples. Results are displayed as ± SEM, with statistical significance where $P < 0.05$. (*N*=3).

5.3.5 Determining the effects of phenolic extracts I and II on human PBMC viability

Human PBMC's were isolated and seeded at a final concentration of 500,000 cells per well. Both extracts were prepared at various concentrations in media and added to each well. After 2, 4 and 6 hours, resazurin solution was added to each well and measured spectrophotometrically at 595 nM.

At all three time-points, extract I significantly reduced PBMC viability at concentrations of 1 and 0.5 mg/mL ($P < 0.0001$). (Figure 29).

With extract II, only a concentration of 1 mg/mL significantly reduced PBMC viability at all three time-points ($P < 0.0001$). (Figure 30).

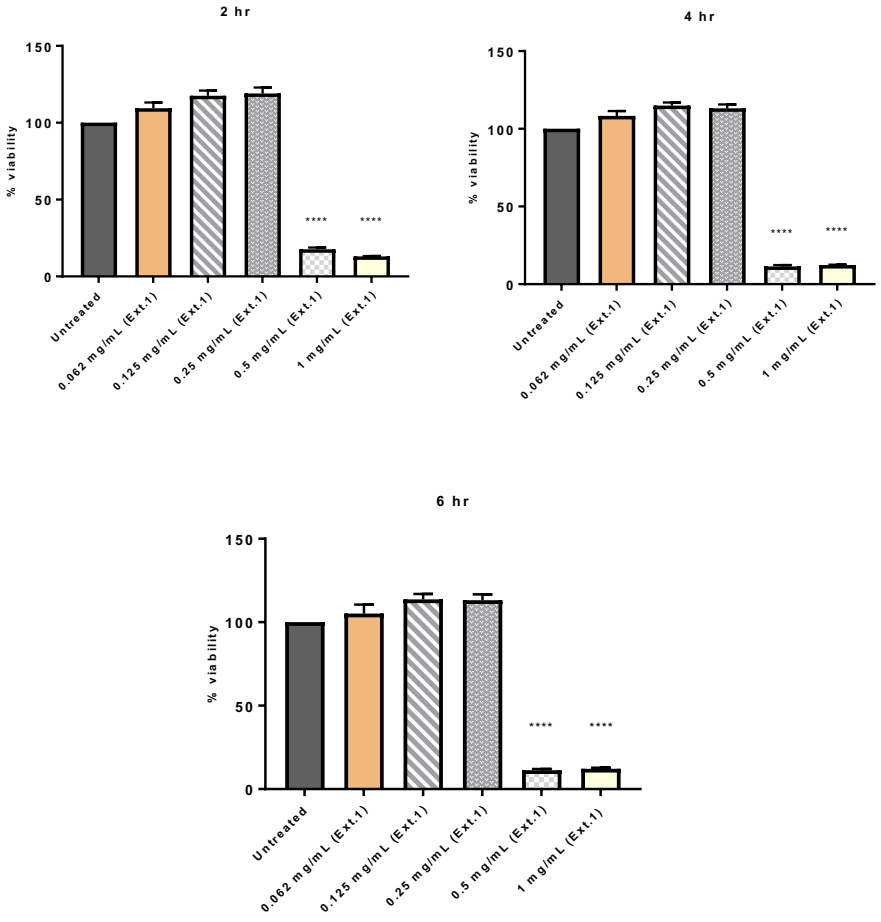


Figure 29. **Effects of phenolic extract I on the viability of human PBMC's**. The resazurin assay was performed after 2, 4 and 6 hour treatments with extract I. **2 hr:** At concentrations of 1 and 0.5 mg/mL, cellular viability was significantly reduced (P< 0.0001). **4 hr**: At concentrations of both 1 and 0.5 mg/mL, cellular viability was significantly reduced (P < 0.0001) **6 hr**: At concentrations of both 1 and 0.5 mg/mL, cellular viability was significantly reduced (P < 0.0001). Statistical analysis was performed using a Dunnett's Test vs untreated samples. Results are displayed as ± SEM, with statistical significance where P < 0.05. (*N*=3).

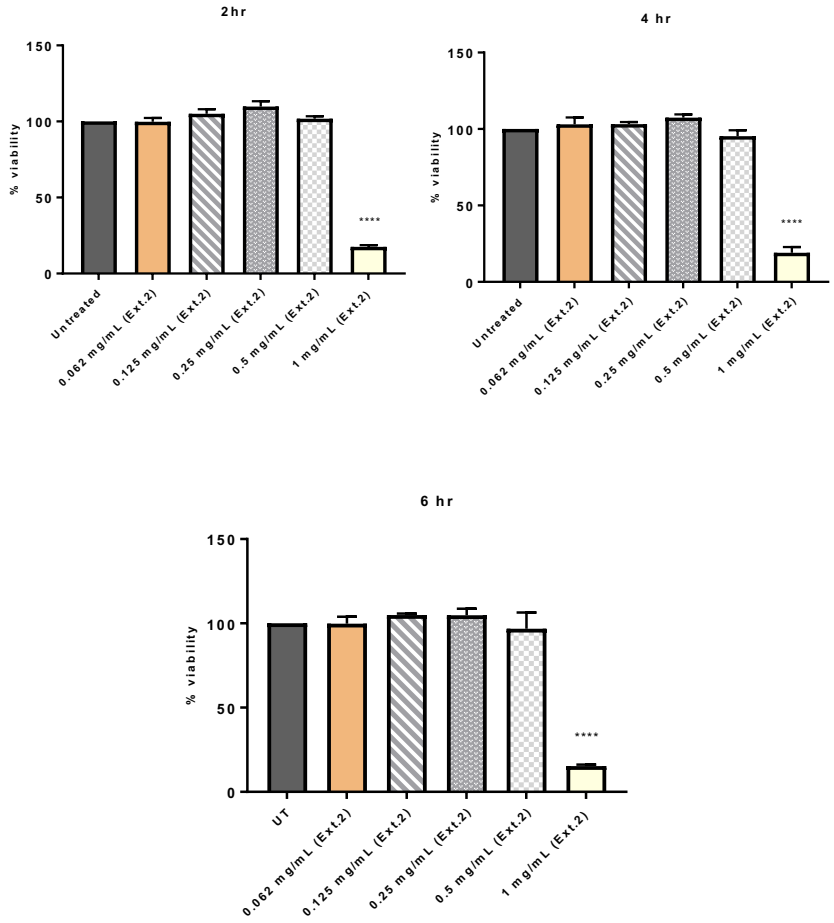


Figure 30. **Effects of phenolic extract II on the viability of human PBMC's.** The resazurin assay was performed after 2, 4 and 6 hour treatments with extract II. **2 hr:** At a concentration of 1 mg/mL, cellular viability was significantly reduced (P< 0.0001). **4 hr**: At a concentration of 1 mg/mL, cellular viability was significantly reduced (P < 0.0001) **6 hr**: At a concentration 1 mg/mL, cellular viability was significantly reduced (P < 0.0001). Statistical analysis was performed using a Dunnett's Test vs untreated samples. Results are displayed as ± SEM, with statistical significance where P < 0.05. (*N*=3).

5.3.5.1 Effects on the viability of human PBMC's treated with phenolic extracts 1 and II following exposure to LPS

Human PBMC's were isolated and seeded at a final concentration of 500,000 cells per well. The cells were then pre-treated with various concentrations of each extract for 3 hours. After this pre-treatment, LPS (20 ng/mL) in fresh media was added to each well. After 2, 4 and 6 hours, resazurin solution was added to each well and measured spectrophotometrically at 595 nM.

After a 2, 4 and 6 hour exposure to LPS, cells pre-treated with extract 1 significantly reduced PBMC viability at concentrations of 1 and 0.5 mg/mL ($P < 0.0001$). (Figure 31).

With cells pre-treated with extract II, only a concentration of 1 mg/mL significantly reduced PBMC viability ($P < 0.0001$). (Figure 32).

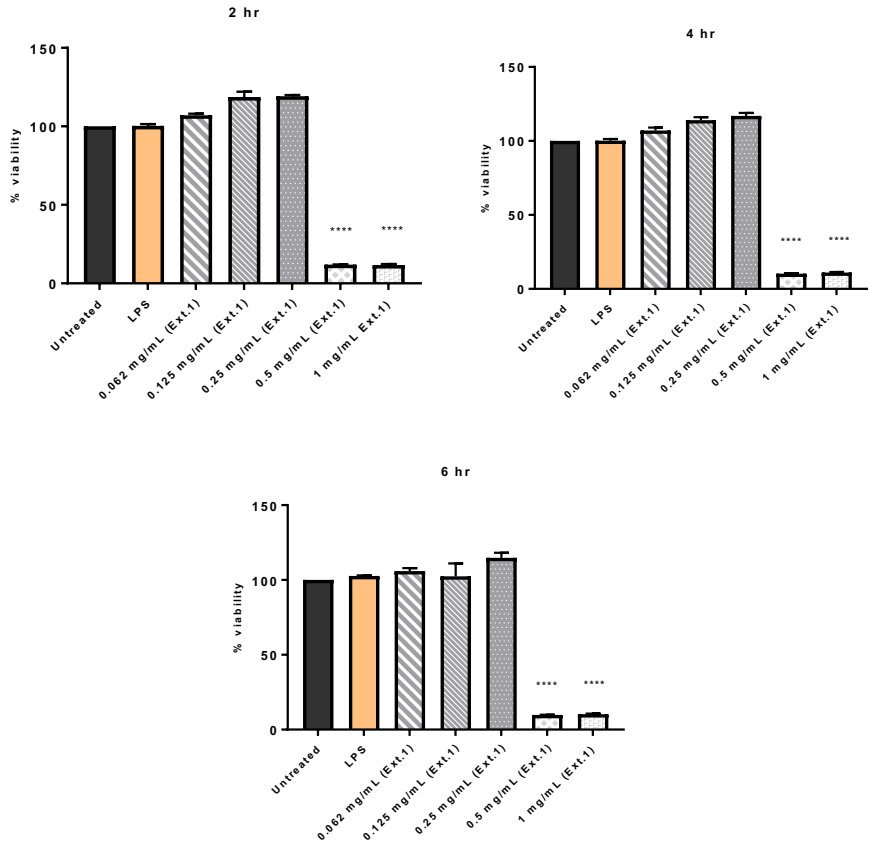


Figure 31. **Effects of phenolic extract I on the viability of human PBMC's after the addition of LPS for 2, 4 and 6 hours**. The resazurin assay was performed after 2, 4 and 6 hour treatments with 20 ng/mL LPS. **2 hr:** At concentrations of 1 and 0.5 mg/mL, cellular viability was significantly reduced ($P < 0.0001$). **4 hr**: At concentrations of both 1 and 0.5 mg/mL, cellular viability was significantly reduced ($P < 0.0001$) **6 hr**: At concentrations of both 1 and 0.5 mg/mL, cellular viability was significantly reduced ($P < 0.0001$). Statistical analysis was performed using a Dunnett's Test vs untreated samples. Results are displayed as ± SEM, with statistical significance where $P < 0.05$. (N=3).

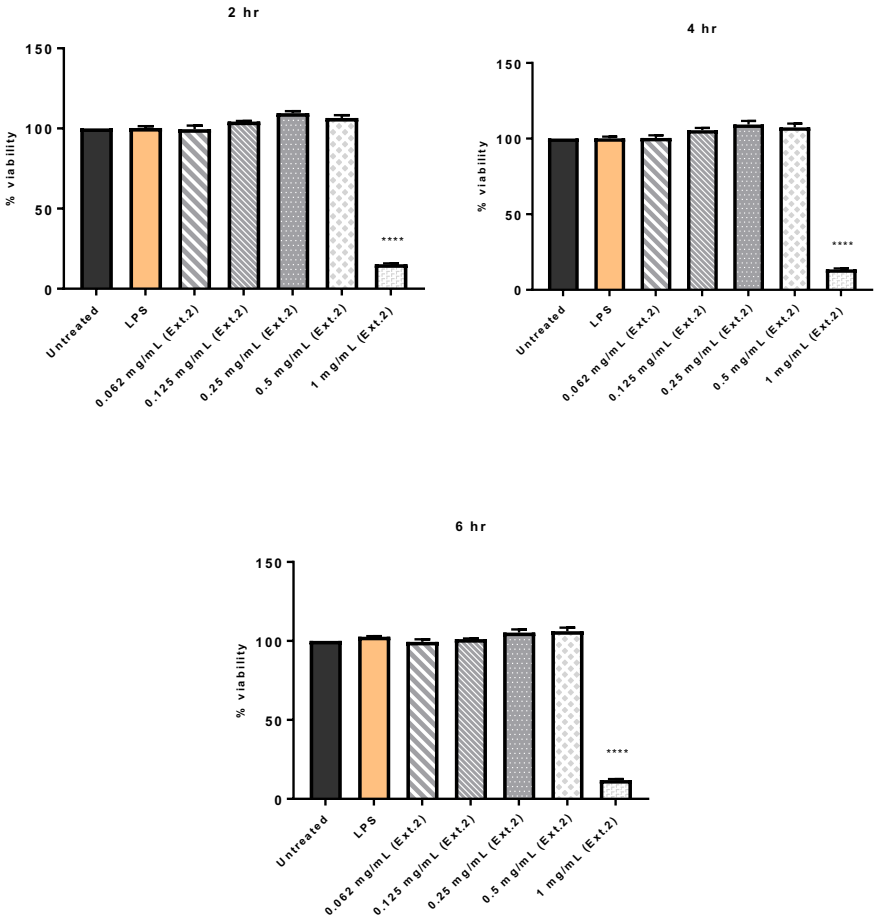


Figure 32. **Effects of phenolic extract II on the viability of human PBMC's after the addition of LPS for 2, 4 and 6 hours**. The resazurin assay was performed after 2, 4 and 6 hour treatments with 20 ng/mL LPS. **2 hr:** At a concentration of 1 mg/mL, cellular viability was significantly reduced (P< 0.0001). **4 hr**: At a concentration of 1 mg/mL, cellular viability was significantly reduced (P < 0.0001) **6 hr**: At a concentration 1 mg/mL, cellular viability was significantly reduced (P < 0.0001). Statistical analysis was performed using a Dunnett's Test vs untreated samples. Results are displayed as ± SEM, with statistical significance where P < 0.05. (*N*=3).

5.3.6 Phenolic extracts I and II significantly inhibit LPS-induced TNF-alpha expression in human PBMC's

The phenolic extracts generated were tested for their ability to inhibit LPS-induced TNF-alpha expression in human PBMCs. Isolated PBMCs were pre-treated with the extracts for 3 hours, before LPS (20 ng/mL) was added for 2 and 6 hours.

After 2 hours post-LPS addition, PBMCs treated with extract I at 0.25, 0.5 and 1 mg/mL had a significant reduction in TNF-alpha expression (P 0.0078; P 0.0031; P 0.0016, respectively) (Figure 33 A). After 6 hours, all five concentrations of extract I: 0.062, 0.125, 0.25, 0.5 and 1 mg/mL, significantly reduced TNF-alpha expression (P 0.0086; P 0.0011; P <0.0001, respectively). (Figure 33 A).

With extract II, PBMCS pre-treated with 0.062, 0.25, 0.5 and 1 mg/mL, all had a significant reduction in TNF-alpha expression after 2 hours with LPS (P 0.0421; P 0.0460; P 0.0010; P 0.0015, respectively) (Figure 33 B). Following 6 hours with LPS, PBMCS treated with extract II at concentrations of 0.125, 0.25, 0.5 and 1 mg/mL had significantly reduced TNF-alpha expression (P 0.0196; P 0.0041; P 0.0002; P 0.0003, respectively) (Figure 33 B).

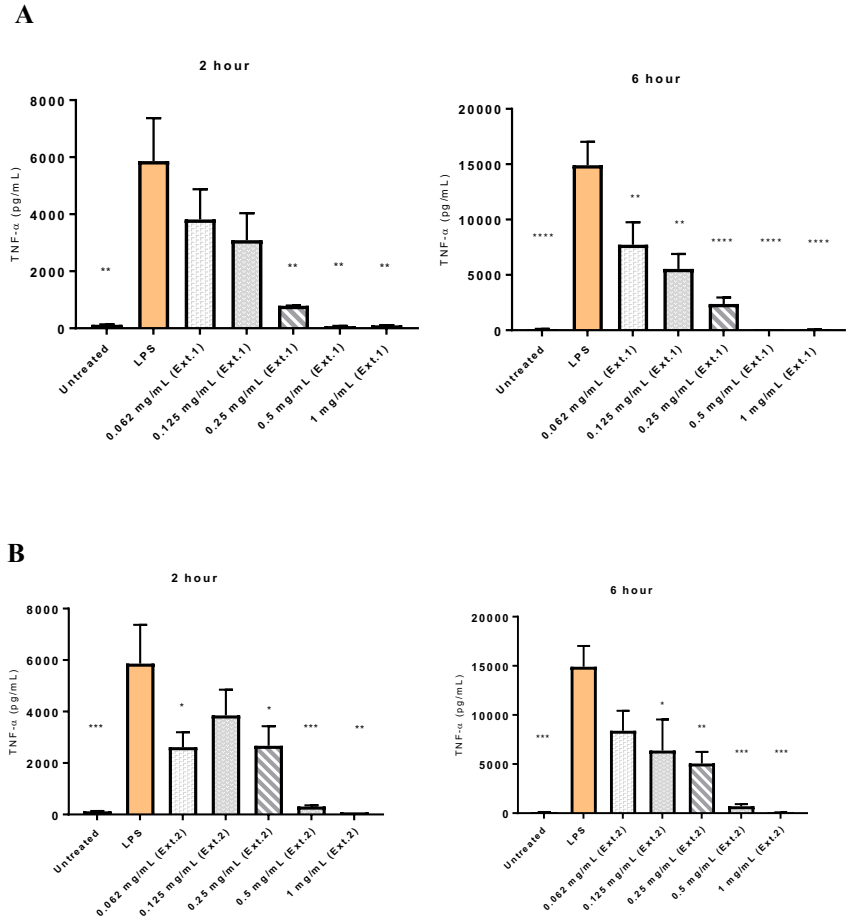


Figure 33. **Effect of extracts I and II on LPS-induced TNF-alpha expression in human PBMCs.** Isolated PBMCs were pre-treated with both extracts at various concentrations for 3 hours. LPS (20 ng/mL) was then added for 2 and 6 hours before supernatants were collected for ELISA analysis. **(A) 2h:** PBMCs treated with extract 1 at 0.25, 0.5 and 1 mg/mL had a significant reduction in TNF-alpha expression (P 0.0078; P 0.0031; P 0.0016, respectively). **6h:** All concentrations of extract 1: 0.062, 0.125, 0.25, 0.5 and 1 mg/mL, significantly reduced TNF-alpha expression (P 0.0086; P 0.0011; P <0.0001, respectively). **(B) 2h**: Extract II at 0.062, 0.25, 0.5 and 1 mg/mL significantly reduced in TNF-alpha expression (P 0.0421; P 0.0460; P 0.0010; P 0.0015, respectively). **6h:** Extract II at concentrations of 0.125, 0.25, 0.5 and 1 mg/mL had significantly reduced TNF-alpha expression (P 0.0196; P 0.0041; P 0.0002; P 0.0003, respectively). Statistical analysis was performed using a Dunnett's Test vs LPS. Results are displayed as ± SEM (*N*=3).

5.3.7 Phenolic extracts I and II significantly inhibit LPS-induced IL-6 expression in human PBMC's

The phenolic extracts generated were tested for their ability to inhibit LPS-induced TNF-alpha expression in human PBMCs. Isolated PBMCs were pre-treated with the extracts for 3 hours, before LPS (20 ng/mL) was added for 2 and 6 hours.

Isolated PBMCs after 2 hours with LPS, had a significant reduction in IL-6 expression after pre-treatments with extract I at 0.5 and 1 mg/mL (P 0.0348). After 4 hours, pre-treatments with extract I at 0.5 and 1 mg/mL again significantly reduced IL-6 (P 0.0010) (Figure 34). However, 6 hours post-LPS addition, all concentrations of extract I significantly reduced IL-6 expression: 0.5 and 1 mg/mL (P < 0.0001); 0.25 mg/mL (P 0.0045); 0.125mg/mL (P 0.0002) and 0.062 mg/mL (P 0.0345) (Figure 34).

With extract II, after 2 hours with LPS, both 1 and 0.5 mg/mL significantly reduced IL-6 (P 0.0066; 0.0087, respectively) (Figure 35). Again, extract II at both 1 and 0.5 mg/mL significantly reduced IL-6 expression after 4 hours with LPS (P 0.0006). After a 6 hour exposure to LPS, extract II significantly reduced IL-6 expression at 0.062, 0.25, 0.5 and 1 mg/mL (P 0.0420; P 0.0233; P 0.0010; P 0.0005, respectively) (Figure 35).

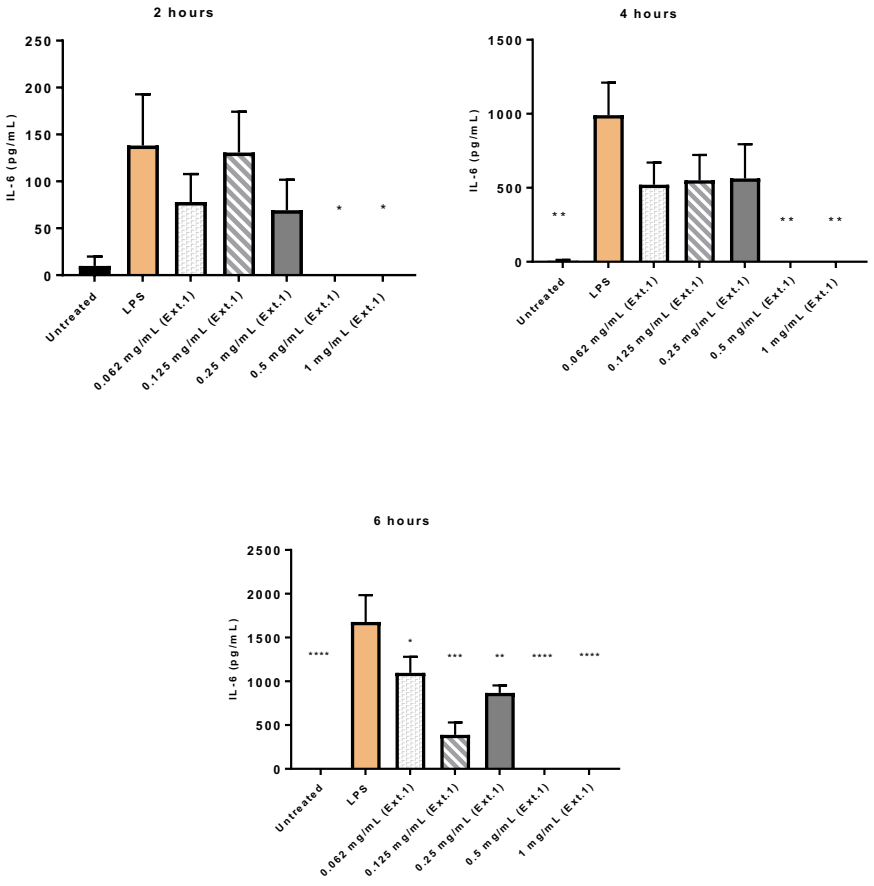


Figure 34. Effect of extract I on LPS-induced expression of IL-6 in human PBMCs. Isolated PBMCs were pre-treated with extract I at various concentrations for 3 hours. LPS (20 ng/mL) was then added for 2, 4 and 6 hours before supernatants were collected for ELISA analysis. **2h**: Concentrations of 0.5 and 1 mg/mL significantly reduced IL-6 expression (P 0.0348). **4h**: Extract I at 0.5 and 1 mg/mL significantly reduced IL-6 expression (P 0.0010). **6h**: Extract I significantly reduced IL-6 expression at 0.062, 0.125, 0.25, 0.5 and 1 mg/mL (P 0.0345; P 0.0002; P 0.0045; P < 0.0001, respectively). Statistical analysis was performed using a Dunnett's Test vs LPS. Results are displayed as ± SEM (N=3).

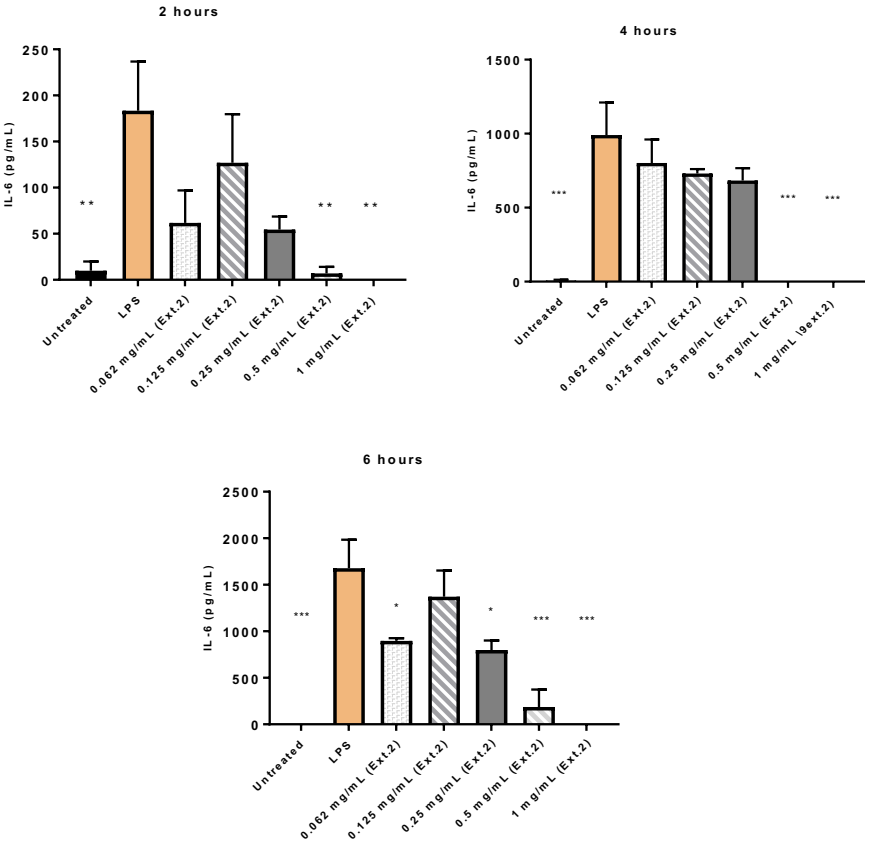


Figure 35. Effect of extract II on LPS-induced IL-6 expression in human PBMCs. Isolated PBMCs were pre-treated with extract II at various concentrations for 3 hours. LPS (20 ng/mL) was then added for 2, 4 and 6 hours before supernatants were collected for ELISA analysis. **2h**: Extract II at both 1 and 0.5 mg/mL significantly reduced IL-6 expression (P 0.0066; 0.0087, respectively). **4h**: At concentrations of 1 and 0.5 mg/mL, extract II significantly reduced IL-6 expression (P 0.0006). **6h**: Extract II significantly reduced IL-6 expression at 0.062, 0.25, 0.5 and 1 mg/mL (P 0.0420; P 0.0233; P 0.0010; P 0.0005, respectively). Statistical analysis was performed using a Dunnett's Test vs LPS. Results are displayed as ± SEM (*N*=3).

5.3.8 Phenolic extracts I and II significantly inhibit LPS-induced IL-12 expression in human PBMC's

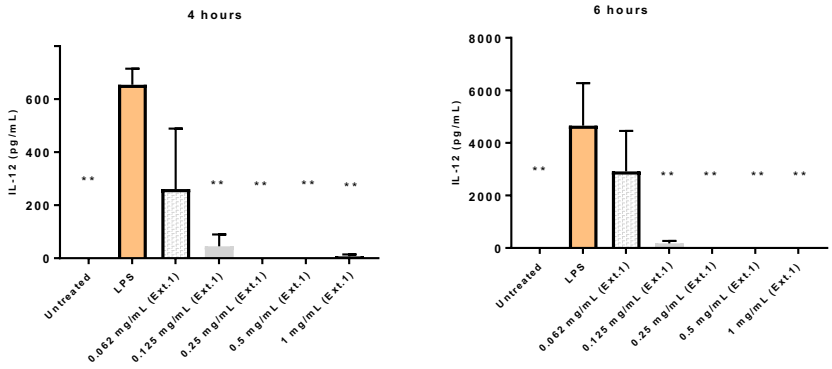
The phenolic extracts generated were tested for their ability to inhibit LPS-induced TNF-alpha expression in human PBMCs. Isolated PBMCs were pre-treated with the extracts for 3 hours, before LPS (20 ng/mL) was added for 2, 4 and 6 hours.

After the addition of LPS for 2 hours, there was no IL-12 expression detected with either extract (data not shown).

After 4 hours, PBMCs pre-treated with extract I at concentrations of 1, 0.5, 0.25 and 0.125 mg/mL, significantly reduced IL-12 expression (P 0.0033; P 0.0030; P 0.0052, respectively) (Figure 36 A). After 6 hours with LPS, extract I again at concentrations of 1, 0.5, 0.25 and 0.125 mg/mL significantly reduced IL-12 expression (P 0.0053; P 0.0071, respectively) (Figure 36 A).

PBMCs pre-treated with extract II followed by the addition of LPS for 4 hours, had a significant reduction in IL-12 expression at concentrations of 1, 0.5 and 0.25 mg/mL (P 0.0281) (Figure 36 B). Following 6 hours with LPS, extract II significantly reduced IL-12 expression at concentrations of 1, 0.5 and 0.25 mg/mL (P 0.0208; P 0.0251, respectively) (Figure 36 B).

A



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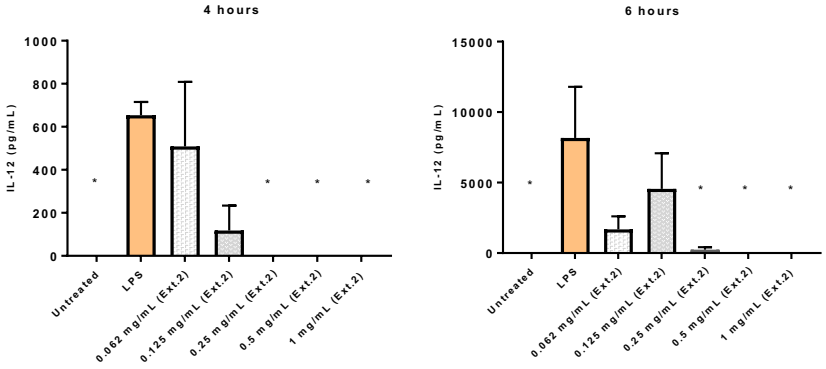

Figure 36. **Effect of extracts I and II on LPS-induced expression of IL-12 in human PBMCs**. Isolated PBMCs were pre-treated with extract 1 at various concentrations for 3 hours. LPS (20 ng/mL) was then added for 4 and 6 hours before supernatants were collected for ELISA analysis. **(A) 4h**: Extract I at 1, 0.5, 0.25 and 0.125 mg/mL significantly reduced IL-12 expression (P 0.0033; P 0.0030; P 0.0052, respectively) **6h:** At concentrations of 1, 0.5, 0.25 and 0.125 mg/mL, extract I significantly reduced IL-12 expression (P 0.0053; P 0.0071, respectively). **(B) 4h**: Extract II at 1, 0.5 and 0.25 mg/mL significantly reduced IL-12 expression (P 0.0281). **6h**: Extract II significantly reduced IL-12 expression at concentrations of 1, 0.5 and 0.25 mg/mL (P 0.0208; P 0.0251, respectively).Statistical analysis was performed using a Dunnett's Test vs LPS. Results are displayed as ± SEM (N=3).

5.4 Discussion

Inflammation has long been a major source of chronic pain in ailments such as osteoarthritis. However, it also has more serious implications in the underlying pathologies of diseases such as cancer, diabetes, inflammatory bowel disease and neurological disorders. Natural products which possess anti-inflammatory activity are currently being investigated as alternatives to chemical drugs without the adverse side effects. This chapter aimed to assess the anti-inflammatory activities of phenolic extracts from Irish rapeseed meal containing SA, along with commercial SA for comparison. PBMCs contain lymphocytes (T cells, B cells, NK cells) and monocytes. Upon stimulation with LPS, monocytes secrete a variety of pro-inflammatory cytokines such as TNF-alpha and IL-12. PBMCs are used to study inflammation which is more representative of the *in-vivo* setting.

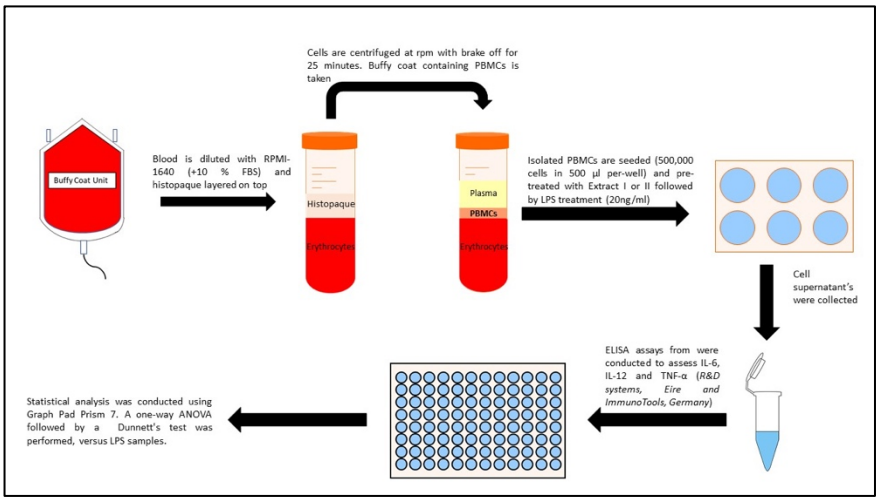

Figure 37. Schematic illustration of the isolation of PBMCs and ELISA analysis

The resazurin assay was used to determine the non-toxic dose of extract I, II and commercial SA. This is necessary to determine if any reduction in cytokine level is due to the anti-inflammatory action of the extracts and not their cytotoxicity. Commercial SA was found to significantly reduce the expression of the inflammatory cytokines TNF-alpha, IL-1β and IL-12. The reduction was significant at non-toxic concentrations of SA both 2 and 6 hours after the addition of LPS. Both IL-8 and IL-6 were tested, however inhibition was only observed at a toxic concentration of 10 mM.

The phenolic extracts generated were then tested for their ability to reduce the expression of pro-inflammatory cytokines. Initially, the resazurin assay was performed for both extracts in order to determine non-toxic concentrations of each. Extract I and II were found to significantly reduce the expression of TNF-alpha, IL-6 and IL-12. Both extracts were also found to reduce IL-8, however only at toxic concentrations.

The results suggest that the extracts and commercial SA are acting as a TLR-4 antagonist, thereby blocking the action of LPS. This is evidenced by the increase in pro-inflammatory cytokines with LPS alone, compared to the decrease observed with the addition of the extracts. The reduction in pro-inflammatory cytokines could also be due to inhibition of NF-κB, which upon translocation to the nucleus results in the production of pro-inflammatory cytokines. Previous studies have shown that commercial SA can prevent activation of

NF-κB, and so this could be one possible mechanism of action (Zhang *et al.*, 2017; Yun *et al.*, 2008). One method which could be used to confirm this would be to perform western blot analysis of inhibitory kappa B (IκBα) or p65 proteins.

The results demonstrate the potential use these extracts might have as anti-inflammatory agents. As mentioned previously, inflammation is involved in numerous diseases. TNF-alpha, along with IL-1β provides a chemotactic stimulus for leukocytes to migrate to the intima (the innermost layer of the artery wall) and adhere. This process is a key step in formation of atherosclerotic plaques, which are a major cause of cardiovascular disease (Ambriz-Pérez *et al.*, 2016). Previous work carried out in Chapter 3 has shown that the extracts also inhibit the ACE-I enzyme, which plays a role in heart health.

Osteoarthritis is the most common form of arthritis, and is expected to be the biggest cause of disability among the general population by the year 2030 (Mathiessen and Conaghan, 2017). Synovial inflammation, termed synovitis, is found at all stages of OA progression, and targeting this inflammation is a promising therapeutic strategy. Numerous pro-inflammatory cytokines are up-regulated in OA, including TNF-alpha and IL-6, resulting in increased inflammation and cartilage degradation (Mathiessen and Conaghan, 2017). Another form of arthritis, rheumatoid arthritis (RA), is also characterised by systemic inflammation and synovitis.

Again, elevated levels of cytokines such as TNF-alpha and IL-6 are observed (Urman *et al.*, 2018). In an animal model of RA, a deficiency in IL-6 was found to inhibit arthritis, highlighting its importance in the pathogenesis of this disease (Ogata *et al.*, 2019).

Systemic inflammation has numerous adverse effects, and this is highlighted by the increased risk of cardiovascular disease in patients with RA. Inhibition of TNF-alpha and IL-6 have shown to improve vascular function and lessen inflammation in patients with RA (Urman *et al.*, 2018). Insulin resistance was also be found to be more prevalent in RA patients compared to the general population, at 54 % compared to 40-45 %, respectively (Urman *et al.*, 2018). TNF-alpha plays a key role in the development of insulin resistance, by decreasing the expression of GLUT4, which is an insulin-dependent glucose transporter. TNF-alpha also plays a key role in the pathogenesis of obesity-induced insulin resistance, with increasing amounts in the systemic circulation, liver and adipocytes (Akash, Rehman and Liaqat, 2018). The levels of TNF-alpha are also higher in obese diabetic patients than lean ones, with levels of 51 % and 21 %, respectively. Crucially, TNF-alpha is also one of the main stimuli involved in apoptosis of pancreatic β-cells.

Given these inflammatory cytokines play key roles in multiple diseases, the extracts generated could be used as therapeutic agents. Current treatments targeting inflammation are seen in Table 9.

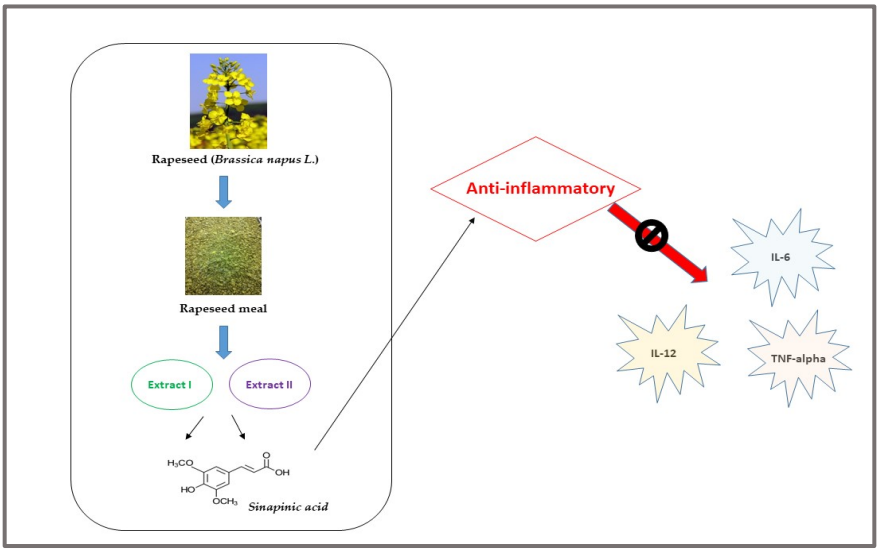

Figure 38. Summary of the anti-inflammatory effects of SA-containing extracts.

Drug type	Examples	Side effects
Traditional non-selective NSAIDs	▪ Ibuprofen ▪ Aspirin	Gastrointestinal disorders Renal toxicity
Selective COX-2 inhibitors	▪ Celecoxib ▪ Rofecoxib ▪ Valdecoxib	Cardiovascular health including congestive heart failure
Synthetic glucocorticoids	▪ Dexamethasone ▪ Betamethasone	GI ulcers; Bleeding; Infection
Conventional disease-modifying anti-rheumatic drugs (cDMARDs)	▪ Methotrexate ▪ Leflunomide	Acceptable toxicity profile
Anti-TNF biologics	▪ Infliximab ▪ Etanercept ▪ Adalimumab ▪ Certolizumab	Infections (tuberculosis, pneumonia) Cardiovascular events
Anti-IL-6 biologics	▪ Tocilizumab ▪ Sarilumab	Infection (Upper respiratory tract, urinary tract)

Table 9. A list of treatments for inflammation and its associated diseases, such as arthritis and inflammatory bowel disease, along with their side effects (Li *et al*. 2017; Ogata *et al*. 2019; Rossi *et al*. 2015).

As seen in Table 9, the use of NSAIDs are associated with adverse side effects, commonly affecting the gastrointestinal system. One reason NSAIDs cause GI damage is due to their inhibition of cyclo-oxygenase (COX) enzymes, which subsequently interferes with the efficacy of the mucous-bicarbonate barrier, negating its protective function (Russell, 2001). These drugs can also have an impact on the surface of the GI system, acting as a topical irritant. The majority of NSAIDS are themselves weak acids, which also plays a contributory role (Russell, 2001). The extracts generated as part of this project offer advantages as a therapeutic agent to treat inflammation. In Chapter 3, the extracts were assessed for their effects on the viability of Caco-2 cells, which serve as a model of the intestinal epithelial barrier. At concentrations which were effective at reducing cytokines such as TNF-alpha and IL-6, there were no significant effects on Caco-2 viability observed. The extracts should not cause damage to the epithelium. The inhibition of COX enzymes also contributes to GI damage, however the extracts generated are cytokine-targeted and so should not interfere with these enzymes. The cardiovascular system is also affected by the use of NSAIDs and anti-TNFs. However, the extracts were shown in this work to possess heart health benefits and act as ACE-I inhibitors which can ameliorate hypertension.

Current cytokine-targeting therapeutics, such as the TNF and IL-6 biologics, are administered via intravenous (I.V) or subcutaneous injection (Ogata *et al.*, 2019). Extract I and II could be incorporated

into food matrices, such as snacks or drinks, which are easily consumed and more patient-friendly. The nutraceutical market in Europe is predicted to register a Compound Annual Growth Rate (CAGR) of 7.5 % from 2019-2024. The reason for this increase is believed to be due an increase in lifestyle-related diseases, aging and increased consumer awareness of the impact dietary factors can play in health and disease. In the nutraceuticals market, functional foods hold the largest market share, which include dairy, bakery, snacks and cereals (Intelligence, 2018). Therefore developing a functional food product containing the extracts would be well received in a well-established market.

In summary, this chapter has highlighted the anti-inflammatory effects of phenolic extracts I and II generated from Irish rapeseed meal. Extracts reduced key pro-inflammatory cytokines involved in the pathogenesis of various autoimmune diseases.

Chapter 6

General discussion

6.1 General discussion

Rapeseed meal is a low-economic value by-product from the rapeseed de-oiling process, and is ordinarily used as animal feed. Rapeseed is rich in bioactive compounds including phenolic acids, the majority of which remain in the meal after processing of the oil. Developing and improving methods to extract these phenolics from rapeseed meal could not only enhance the economic value of this resource, but also encompasses the idea of a 'circular economy'. This ensures that resources which are normally considered waste are recycled back into the economy (Velis, 2015). The fitness and sports nutrition industry encompasses this concept through the valorisation of a by-product of cheese manufacture, whey. Whey protein products are widely available and extremely popular, with a variety of products available such as powders, shakes and snack bars.

The first aim of this work was to generate extracts containing the phenolic sinapinic acid from Irish rapeseed meal. An initial methodology was chosen based on the phenolic acid composition of rapeseed meal, in terms of both its free and esterified forms. This resulted in the generation of an extract containing 5.31 mg of SA per 100 mg of extract. Further modifications to the initial methodology were performed, and this resulted in the generation of an extract with an SA content of 0.569 mg/mL (57 %). Previous studies have reported SA in rapeseed to be in the ranges of 170-454 µg/g and 0.11-.0.59 mg/g (Nićiforović and Abramovič, 2014; Khattab *et al.*,

2010). The modified methodology used in this study is therefore an efficient way to ensure that maximum levels of SA are retrieved from rapeseed meal in one extraction. The generation of these extracts was an important and novel aspect of the project moving forward, as previous studies examining the bioactivities of SA have used a chemically manufactured version.

The extracts and commercial SA were found to inhibit the ACE-I enzyme, which plays a role in hypertension (Figure 39). ACE-I inhibitors work by competitive inhibition of the ACE-I enzyme, blocking the formation of angiotensin II. This results in dilation of veins and arteries. The level of inhibition of the extracts was on a par with the known ACE-I inhibitor, Captopril. When assayed at 1 mg/mL, Captopril displayed 96 % ± 0.5 ACE-I inhibition, compared to extracts 1 and II at 97 % ± 4.1 and 91 % ± 3.6, respectively. Inhibition of ACE-1 was previously shown to play a role in the development of diabetes and kidney disease. Hypertensive patients with T2DM who received ACE-I inhibitors had a significant reduction in cardiovascular events; while evidence is also emerging to suggest that ACE-I inhibition plays a role in kidney disease (Hao *et al.*, 2014; Zeier, 2018). One possible mechanism by which the extracts could inhibit the ACE-I enzyme is through the carboxylic acid functional group present in SA. This acts as the ligand which binds to the zinc ion in the active site of the ACE-I enzyme. This is the same mechanism of action as Enalapril, a drug used in the clinical management of hypertension (Shionoiri, Shigemasa and Takasaki,

1997). Initial studies examining plasma levels following consumption of cereal meals and cranberry juice indicate that SA is bioavailable, and quickly absorbed from the small intestine, particularly when in its free form (Hameed, Aydin and Basaran, 2016). It has been shown that the esterification of phenolics impairs their absorption, with free phenolic acids being 10 to 17 times more bioavailable in humans (Hameed, Aydin and Basaran, 2016). Approximately 70-96 % of the phenolic acids present in rapeseed meal are esterified, which could also explain why dietary intake is insufficient to exert beneficial effects (Vuorela, 2005). The methodology used to generate the extracts involves hydrolysis of esterified SA, and theoretically should improve the bioavailability of the extracts. *In-vivo* studies are crucial moving forward, to determine their bioavailability, along with their efficacy and toxicity.

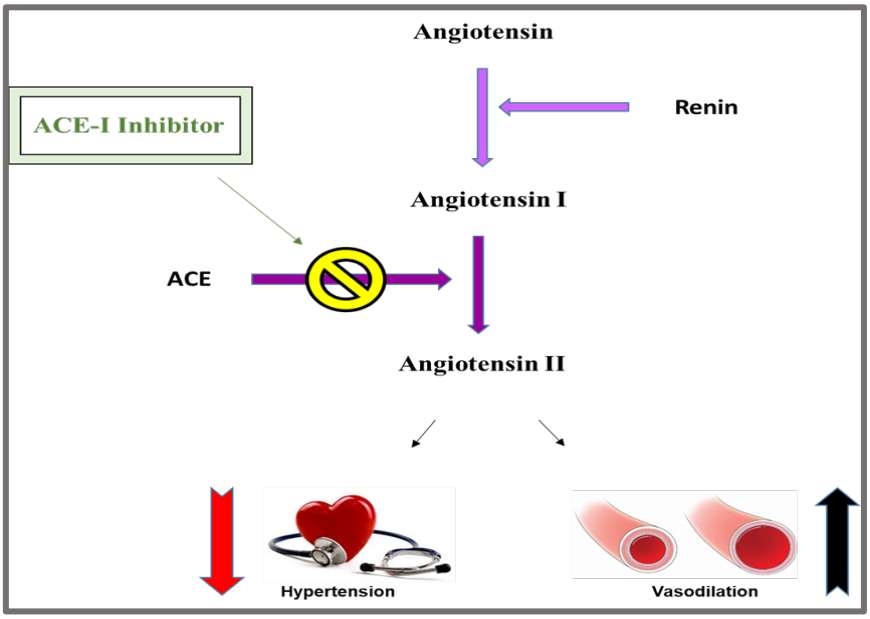

Figure 39. Summary of the ACE-I pathway and its inhibition.

Chronic inflammation is well-established in the underlying pathology of numerous diseases, including cardiovascular disease, diabetes and obesity (Figure 40). Both of the SA-containing extracts, along with commercial SA, were found to significantly reduce the expression of pro-inflammatory cytokines including TNF-alpha, IL-6 and IL-12. Recent studies have made significant progress in elucidating the mechanisms linking inflammation with hypertension. Studies have shown that inhibition of TNF-alpha leads to decreased blood pressure and inflammation in animal models of metabolic syndrome and pre-eclampsia (De Miguel *et al.*, 2015). The levels of IL-6 have also been shown to be elevated in hypertensive conditions, including pulmonary hypertension. Anti-IL-6 therapy has been successfully used in a one-patient trial with severe pulmonary arterial hypertension (Furuya, Satoh and Kuwana, 2010).

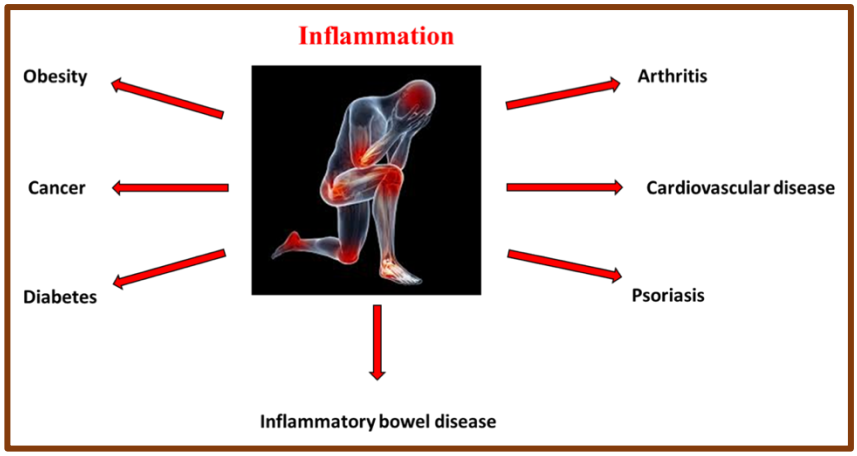

Figure 40. Summary of the diseases in which inflammation plays a role

The mechanisms by which the extracts and commercial SA exert their anti-inflammatory effects have not been investigated. However, based on evidence from previous studies and the structures of other anti-inflammatory bioactive compounds, we suggest possible mechanisms of action (Figure 41). The first mechanism of action involves inhibition of NF-κB, a nuclear transcription factor which plays a key role in the pathogenesis of inflammation. Previous studies have shown that treatment with SA inhibited the nuclear translocation of p65 and p50, thereby preventing transcription initiation by NF-κB (Schmitz and Baeuerle, 1991; Pahl, 1999; Yun *et al.*, 2008). Sinapinic acid was also found to inhibit the LPS-induced degradation of IκB-α, preventing the release of NF-κB (Yun *et al.*, 2008). Extensive studies involving Curcumin have identified mechanisms by which it inhibits TNF-α. Curcumin has also been shown to down-regulate NF-κB activation and inhibit TNF (Aggarwal, Gupta and Sung, 2013). A potential mechanism of action has recently been proposed by Edwards et al. 2017. By generating curcumin analogs which could either resist or undergo spontaneous oxidation, they found the oxidised analogs were potent inhibitors of NF-κB. These oxidative metabolites bind to and inhibit IKKβ, thereby preventing NF-κB translocation (Edwards *et al.*, 2017). Both curcumin and SA have similar structures, such as phenolic hydroxyl groups, and are both hydroxycinnamic acid derivatives (Alam *et al.*, 2016). Studies could be performed with our SA-containing extracts and commercial SA to see if a similar mechanism is occurring.

The second potential mechanism involves inhibiting LPS signalling through toll-like receptor 4. The activation of TLR-4 starts a signalling cascade which results in NF- κB transcription and inflammatory cytokine production. Studies involving curcumin have shown that it can inhibit both ligand-dependent and independent dimerization of TLR-4, while also reducing the expression levels of TLR. Future work is necessary in order to elucidate which mechanism of action is occurring with our extracts and commercial SA. This could be done by examining GFP-tagged Re1A (p65) in macrophage cells stimulated with LPS to examine NF- κB translocation (Ernst, Vayttaden and Fraser, 2018). PCR analysis could determine if the extracts and SA reduce the expression levels of TLR-4, while molecular docking studies would also be useful to determine if the binding site of SA overlaps with that of LPS. *In-vivo* studies are also necessary to determine the efficacy of the extracts, and there have been discussions with a research group in Israel in regard to studying the effects of the extracts in an *in-vivo* model of inflammation.

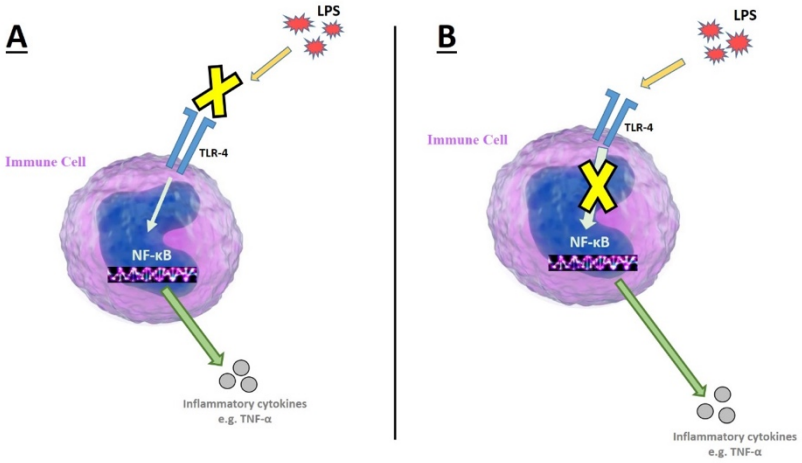

Figure 41. Potential mechanisms of TNF-α inhibition by phenolic extracts and commercial SA. (A) They could be acting as a toll-like receptor-4 antagonist (B) they could inhibit the translocation and transcription of the nuclear transcription factor NF-κB. Both mechanisms ultimately result in the inhibition of pro-inflammatory cytokine production.

Experiments were also carried out to determine if the extracts and commercial SA effect GLUT4 expression. Our results showed no effects on GLUT4 expression in the three cell lines after treatments with the extracts and commercial SA. However, there were limitations to this work in terms of the *in-vitro* models used, and there were no *in-vivo* studies performed. Skeletal muscle is one of the key insulin-target tissues, hence the H9c2 myoblast cell line was used in this study. However, future studies would benefit from experimentation with the L6 myoblast cell line (Yap *et al.*, 2007). The L6 myoblast cells can be differentiated into myotubes by reducing the fetal bovine serum to 2 % and display insulin-dependent

glucose uptake and GLUT4 translocation. This is a promising in-vitro model to identify the possible anti-diabetic effects of the extracts (Yap *et al.*, 2007). Chronic hyperglycemia is a common characteristic of T2DM and inhibition of carbohydrate-hydrolysing enzymes could help to prevent these symptoms by decreasing post-prandial glucose levels (Padilla-Camberos *et al.*, 2014). Phenolic extracts from *Citrus limetta* inhibited both α-amylase and α-glucosidase (Padilla-Camberos *et al.*, 2014). Simple assays could be performed with our extracts to determine if they can inhibit these enzymes.

Our diet plays a crucial role in the development of diseases such as cardiovascular disease, inflammation, diabetes and obesity. Equally, so too can our diet aide in disease prevention (Ozen *et al.*, 2014). Consumers today are more aware of the importance of a healthy, balanced diet, and this has resulted in increased sales of functional foods/ nutraceuticals. These are defined as a food or food ingredients which can prevent disease development (Inan, 2019). The nutraceutical market in Europe is predicted to register a Compound Annual Growth Rate (CAGR) of 7.5 % from 2019-2024. The reason for this increase is due to an increase in lifestyle-related diseases, aging and increased consumer awareness of the impact dietary factors can play in health and disease. In the nutraceuticals market, functional foods hold the largest market share, which include dairy, bakery, snacks and cereals (Intelligence, 2018). Current pharmacological agents used to treat inflammation and hypertension

are associated with various side effects, and have undesirable methods of administration. For example, current cytokine-targeting therapeutics, such as the TNF and IL-6 biologics, are administered via I.V or subcutaneous injection (Ogata *et al.*, 2019). Natural extracts possessing anti-inflammatory and hypertensive properties could eliminate these effects. Supplements, snacks or drinks containing the extracts would provide an amenable method of disease prevention for those of all ages, which are easily consumed and more patient friendly. The extracts could also be consumed pro-actively to prevent the onset of inflammation and to maintain normal blood pressure. There is already a well-established market for these products, including products such as Benecol® which contains plant sterols to lower cholesterol, or Yakult- a probiotic yogurt drinks to maintain healthy digestion.

As discussed in chapter 3, there are already numerous clinical trials involving phenolic acids, paving the way for clinical studies with extracts like those prepared in this study. If a product is to be accepted as a functional food ingredient, the EU has a strict regulation in place- EC Regulation 1924/2006 (Moors, 2012). Under this regulation, foods or their constituents can be classified under three different 'claims', one of which is 'reduction of disease risk claim'. There must be a 'dossier with full details of the food/constituent and substantial scientific evidence verifying the claim' (Moors, 2012). Any data obtained from *in-vitro* and *in-vivo* studies may only be used as supplementary evidence.

References

Aggarwal, B. B., Gupta, S. C. and Sung, B. (2013) 'Curcumin: an orally bioavailable blocker of TNF and other pro-inflammatory biomarkers', *British Journal of Pharmacology,* 169(8), pp. 1672-1692.

Ainsworth, E. A. and Gillespie, K. M. (2007) 'Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent', *Nature Protocols,* 2(4), pp. 875-7.

Akash, M. S. H., Rehman, K. and Liaqat, A. (2018) 'Tumor Necrosis Factor-Alpha: Role in Development of Insulin Resistance and Pathogenesis of Type 2 Diabetes Mellitus', *Journal of Cellular Biochemistry,* 119(1), pp. 105-110.

Al Shukor, N., Van Camp, J., Gonzales, G. B., Staljanssens, D., Struijs, K., Zotti, M. J., Raes, K. and Smagghe, G. (2013) 'Angiotensin-converting enzyme inhibitory effects by plant phenolic compounds: a study of structure activity relationships', *Journal of Agricultural and Food Chemistry,* 61(48), pp. 11832-11839.

Alam, M. A., Subhan, N., Hossain, H., Hossain, M., Reza, H. M., Rahman, M. M. and Ullah, M. O. (2016) 'Hydroxycinnamic acid derivatives: a potential class of natural compounds for the management of lipid metabolism and obesity', *Nutrition & Metabolism,* 13(27), pp. 1-13.

Ambriz-Pérez, D. L., Leyva-López, N., Gutierrez-Grijalva, E. P. and Heredia, J. B. (2016) 'Phenolic compounds: Natural alternative in inflammation treatment. A Review', *Cogent Food & Agriculture,* 2(1), pp. 1-14.

Balaji, C., Muthukumaran, J. and Nalini, N. (2015) 'Effect of sinapic acid on 1,2 dimethylhydrazine induced aberrant crypt foci, biotransforming bacterial enzymes and circulatory oxidative stress status in experimental rat colon carcinogenesis', *Bratisl Lek Listy,* 116(9), pp. 560-566.

Bernstein, K. E., Ong, F. S., Blackwell, W. L., Shah, K. H., Giani, J. F., Gonzalez-Villalobos, R. A., Shen, X. Z., Fuchs, S. and Touyz, R. M. (2013) 'A modern understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme', *Pharmacological Reviews,* 65(1), pp. 1-46.

Besancon, A., Goncalves, T., Valette, F., Dahllof, M. S., Mandrup-Poulsen, T., Chatenoud, L. and You, S. (2018) 'Oral histone deacetylase inhibitor synergises with T cell targeted immunotherapy to preserve beta cell metabolic function and induce stable remission of new-onset autoimmune diabetes in NOD mice', *Diabetologia,* 61(2), pp. 389-398.

Bird, I. M. (1989) 'High performance liquid chromatography: principles and clinical applications', *British Medical Journal,* 299(6702), pp. 783-787.

Bodas, M., Mazur, S., Min, T. and Vij, N. (2018) 'Inhibition of histone-deacetylase activity rescues inflammatory cystic fibrosis lung disease by modulating innate and adaptive immune responses', *Respiratory Research,* 19(1), pp. 1-12.

Bommer, C., Sagalova, V., Heesemann, E., Manne-Goehler, J., Atun, R., Bärnighausen, T., Davies, J. and Vollmer, S. (2018) 'Global Economic Burden of Diabetes in Adults: Projections From 2015 to 2030', *Diabetes Care,* 41(5), pp. 963-970.

Booth, E. J. (2004) 'Extraction and Refining.', in Gunstone, F.D. (ed.) *Rapeseed and Canola Oil. Production, Processing, Properties and Uses*, Blackwell Publishing Ltd., pp. 17-29.

Bruice, P. Y. (2004) 'Proton Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy', *Organic Chemistry*: Pearson, (14) pp. 526-592.

Brunet, A. and Berger, S. L. (2014) 'Epigenetics of Aging and Aging-related Disease', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences,* 69(Suppl 1), pp. S17-S20.

Bryant, N. J., Govers, R. and James, D. E. (2002) 'Regulated transport of the glucose transporter GLUT4', *Nature Reviews Molecular Cell Biology,* 3(4), pp. 267-77.

Buchholz, T. and Melzig, M. F. (2015) 'Polyphenolic Compounds as Pancreatic Lipase Inhibitors', *Planta Medica,* 81(10), pp. 771-83.

Cai, R. and Arntfield, S. D. (2001) 'A rapid high-performance liquid chromatographic method for the determination of sinapine and sinapic acid in canola seed and meal', *Journal of the American Oil Chemists' Society,* 78(9), pp. 903-910.

Canada, C. C. o. (2014) '*Steps in Oil and Meal Processing'*. Available at: http://www.canolacouncil.org/oil-and-meal/what-is-canola/how-canola-is-processed/steps-in-oil-and-meal-processing/. (Accessed: 09/11/2015).

Chen, C. (2016) 'Sinapic Acid and Its Derivatives as Medicine in Oxidative Stress-Induced Diseases and Aging', *Oxidative Medicine and Cellular Longevity,* (2016), pp.1-10.

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X. and Zhao, L. (2018) 'Inflammatory responses and inflammation-associated diseases in organs', *Oncotarget,* 9(6), pp. 7204-7218.

Cherng, Y. G., Tsai, C. C., Chung, H. H., Lai, Y. W., Kuo, S. C. and Cheng, J. T. (2013) 'Antihyperglycemic action of sinapic acid in diabetic rats', *Journal of Agricultural and Food Chemistry,* 61(49), pp. 12053-12059.

Cheynier, V., Comte, G., Davies, K. M., Lattanzio, V. and Martens, S. (2013) 'Plant phenolics: Recent advances on their biosynthesis, genetics, and ecophysiology', *Plant Physiology and Biochemistry,* 72(2013), pp. 1-20.

Cho, H. M., Seok, Y. M., Lee, H. A., Song, M. and Kim, I. (2018) 'Repression of Transcriptional Activity of Forkhead Box O1 by Histone Deacetylase Inhibitors Ameliorates Hyperglycemia in Type 2 Diabetic Rats', *International Journal of Molecular Sciences,* 19(11), pp. 1-17.

Chung, B., Matak, P., McKie, A. T. and Sharp, P. (2007) 'Leptin increases the expression of the iron regulatory hormone hepcidin in HuH7 human hepatoma cells', *Journal of Nutrition,* 137(11), pp. 2366-2370.

Cvjetko, M., Lepojević, Z., Zeković, Z., Vidović, S. and Milošević, S. 'Antioxidant properties of rapeseed' (2009) *Modern technologies and economic development*, 19(2009) pp.27-33.

Dai, J. and Mumper, R. J. (2010) 'Plant phenolics: extraction, analysis and their antioxidant and anticancer properties', *Molecules,* 15(10), pp. 7313-52.

De Miguel, C., Rudemiller, N. P., Abais, J. M. and Mattson, D. L. (2015) 'Inflammation and hypertension: new understandings and potential therapeutic targets', *Current Hypertension Reports,* 17(1), pp. 507-507.

Dinarello, C. A. (2011) 'Interleukin-1 in the pathogenesis and treatment of inflammatory diseases', *Blood,* 117(14), pp. 3720-32.

Edwards, R. L., Luis, P. B., Varuzza, P. V., Joseph, A. I., Presley, S. H., Chaturvedi, R. and Schneider, C. (2017) 'The anti-inflammatory activity of curcumin is mediated by its oxidative metabolites', *Journal of Biological Chemistry,* 292(52), pp. 21243-21252.

Ernst, O., Vayttaden, S. J. and Fraser, I. D. C. (2018) 'Measurement of NF-κB Activation in TLR-Activated Macrophages', *Methods in Molecular Biology (Clifton, N.J.),* 1714, pp. 67-78.

Eroğlu, C., Kurar, E., Avcı, E. and Vural, H. (2017) 'Investigation of Apoptotic Effect of Sinapic Acid in Hep3B and HepG2 Human Hepatocellular Carcinoma Cells', *Proceedings,* 1(10) pp.1-1.

Furuya, Y., Satoh, T. and Kuwana, M. (2010) 'Interleukin-6 as a potential therapeutic target for pulmonary arterial hypertension', *International Journal of Rheumatology,* 2010, pp. 1-8.

Gaspar, A., Martins, M., Silva, P., Garrido, E. M., Garrido, J., Firuzi, O., Miri, R., Saso, L. and Borges, F. (2010) 'Dietary Phenolic Acids and Derivatives. Evaluation of the Antioxidant Activity of Sinapic Acid and Its Alkyl Esters', *Journal of Agricultural and Food Chemistry,* 58(21), pp. 11273-11280.

Goncalves, S. and Romano, A. (2017) 'Inhibitory properties of phenolic compounds against enzymes linked with human diseases', *InTech Open,* Chapter 6, pp. 99-118.

González, L. T., Minsky, N. W., Espinosa, L. E. M., Aranda, R. S., Meseguer, J. P. and Pérez, P. C. (2017) 'In vitro assessment of hepatoprotective agents against damage induced by acetaminophen and CCl(4)', *BMC Complementary and Alternative Medicine,* 17(1), pp. 39-39.

Hameed, H., Aydin, S. and Basaran, N. (2016) 'Sinapic acid: Is it safe for humans', *Journal of Pharmaceutical Sciences,* 41, pp. 39-49.

Hao, G., Wang, Z., Guo, R., Chen, Z., Wang, X., Zhang, L. and Li, W. (2014) 'Effects of ACEI/ARB in hypertensive patients with type 2 diabetes mellitus: a meta-analysis of randomized controlled studies', *BMC Cardiovascular Disorders,* 14(1), pp. 148.

Harbaum-Piayda, B., Oehlke, K., Sönnichsen, F. D., Zacchi, P., Eggers, R. and Schwarz, K. (2010) 'New polyphenolic compounds in commercial deodistillate and rapeseed oils', *Food Chemistry,* 123(3), pp. 607-615.

Hasler, C. M. (2002) 'Functional foods: benefits, concerns and challenges-a position paper from the american council on science and health', *Journal of Nutrition,* 132(12), pp. 3772-81.

Hauner, H., Rohrig, K., Spelleken, M., Liu, L. S. and Eckel, J. (1998) 'Development of insulin-responsive glucose uptake and GLUT4 expression in differentiating human adipocyte precursor cells', *International Journal of Obesity and Related Metabolic Disorders,* 22(5), pp. 448-53.

Herman, L. L. and Bashir, K. (2019) *Angiotensin Converting Enzyme Inhibitors (ACEI)*. StatPearls (Internet): StatPearls Publishing. Available at: https://www.ncbi.nlm.nih.gov/books/NBK431051/ (Accessed: 03/03 2019).

Ho, C. S., Lam, C. W., Chan, M. H., Cheung, R. C., Law, L. K., Lit, L. C., Ng, K. F., Suen, M. W. and Tai, H. L. (2003) 'Electrospray ionisation mass spectrometry: principles and clinical applications', *Clinical Biochemist Reviews,* 24(1), pp. 3-12.

Hsu, C. L. and Yen, G. C. (2007) 'Effects of flavonoids and phenolic acids on the inhibition of adipogenesis in 3T3-L1 adipocytes', *Journal of Agricultural and Food Chemistry,* 55(21), pp. 8404-10.

Huang, D., Ou, B. and Prior, R. L. (2005) 'The chemistry behind antioxidant capacity assays', *Journal of Agricultural and Food Chemistry,* 53(6), pp. 1841-56.

Inan, S. (2019) 'The Potential Role of Nutraceuticals in Inflammation and Oxidative Stress', *Nutraceuticals- Past, Present and Future*. IntechOpen, pp. 1-20.

Intelligence, M. (2018) *European Nutraceutical Food Market- Growth, Trends and Forecast (2019-2024)*. Available at: https://www.mordorintelligence.com/industry-reports/europe-nutraceutical-market (Accessed: 20/08 2019).

Irakli, M., Kleisiaris, F., Kadoglidou, K. and Katsantonis, D. (2018) 'Optimizing Extraction Conditions of Free and Bound Phenolic Compounds from Rice By-Products and Their Antioxidant Effects', *Foods,* 7(6), pp. 1-12.

Jeong, H. S., Kim, H. Y., Ahn, S. H., Oh, S. C., Yang, I. and Choi, I. G. (2013) 'Effects of combination processes on the extraction of pectins from rapeseed cake (Brassica napus L.)', *Food Chemistry,* 139(1-4), pp. 9-15.

Kahn, S. E., Hull, R. L. and Utzschneider, K. M. (2006) 'Mechanisms linking obesity to insulin resistance and type 2 diabetes', *Nature,* 444(7121), pp. 840-846.

Kanchana, G., Shyni, W. J., Rajadurai, M. and Periasamy, R. (2011) 'Evaluation of Antihyperglycemic Effect of Sinapic Acid in Normal and Streptozotocin-Induced Diabetes in Albino Rats', *Global Journal of Pharmacology,* 5(1), pp. 33-39.

Khattab, R., Eskin, M., Aliani, M. and Thiyam, U. (2010) 'Determination of Sinapic Acid Derivatives in Canola Extracts Using High-Performance Liquid Chromatography', *Journal of the American Oil Chemist's Society,* 87(2), pp. 147-155.

Khoddami, A., Wilkes, M. A. and Roberts, T. H. (2013) 'Techniques for analysis of plant phenolic compounds', *Molecules,* 18(2), pp. 2328-75.

King, P. J., Ma, G., Miao, W., Jia, Q., McDougall, B. R., Reinecke, M. G., Cornell, C., Kuan, J., Kim, T. R. and Robinson, W. E., Jr. (1999) 'Structure-activity relationships: analogues of the dicaffeoylquinic and dicaffeoyltartaric acids as potent inhibitors of human immunodeficiency virus type 1 integrase and replication', *Journal of Medicinal Chemistry,* 42(3), pp. 497-509.

Klockeman, D., Toledo, R. and Sims, K. (1997) 'Isolation and Characterization of Defatted Canola Meal Protein', *Journal of Agricultural and Food Chemistry,* 45(10), pp. 3867-3870.

Koshihara, Y., Neichi, T., Murota, S., Lao, A., Fujimoto, Y. and Tatsuno, T. (1984) 'Caffeic acid is a selective inhibitor for leukotriene biosynthesis', *Biochimica et Biophysica Acta,* 792(1), pp. 92-7.

Kozlowska, H., Naczk, M., Shahidi, F. and Zadernowski, R. (1990) 'Phenolic Acids and Tannins in Rapeseed and Canola', in Shahidi, F. (ed.) *Canola and Rapeseed*: Springer US, pp. 193-210.

Krygier, K., Sosulski, F. and Hogge, L. (1982) 'Free, esterified, and insoluble-bound phenolic acids. 1. Extraction and purification procedure', *Journal of Agricultural and Food Chemistry,* 30 (2), pp. 330-334.

Lee, J.-Y. (2018) 'Anti-inflammatory effects of sinapic acid on 2, 4, 6-trinitrobenzenesulfonic acid-induced colitis in mice', *Archives of Pharmacal Research,* 41(2), pp. 243-250.

Li, J. and Guo, Z. (2016) 'Identification and quantification of phenolic compounds in rapeseed originated lecithin and antioxidant activity evaluation', *LWT-Food Science and Technology,* 73, pp. 397-405.

Li, X., Lin, J., Ding, X., Xuan, J., Hu, Z., Wu, D., Zhu, X., Feng, Z., Ni, W. and Wu, A. (2019) 'The protective effect of sinapic acid in osteoarthritis: In vitro and in vivo studies', *Journal of Cellular and Molecular Medicine,* 23(3), pp. 1940-1950.

Liu, M., Li, Y. G., Zhang, F., Yang, L., Chou, G. X., Wang, Z. T. and Hu, Z. B. (2007) 'Chromatographic fingerprinting analysis of Danshen root (Salvia miltiorrhiza Radix et Rhizoma) and its preparations using high performance liquid chromatography with diode array detection and electrospray mass spectrometry (HPLC-DAD-ESI/MS)', *Journal of Separation Science,* 30(14), pp. 2256-67.

Liu, Q., Wu, L., Pu, H., Li, C. and Hu, Q. (2012) 'Profile and distribution of soluble and insoluble phenolics in Chinese rapeseed (Brassica napus)', *Food Chemistry,* 135(2), pp. 616-22.

Lomascolo, A., Uzan-Boukhris, E., Sigoillot, J. C. and Fine, F. (2012) 'Rapeseed and sunflower meal: a review on biotechnology status and challenges', *Applied Microbiology and Biotechnology,* 95(5), pp. 1105-14.

López-Martínez, L. M., Santacruz-Ortega, H., Navarro, R.-E., Sotelo-Mundo, R. R. and González-Aguilar, G. A. (2015) 'A ^{1}H NMR Investigation of the Interaction between Phenolic Acids Found in Mango (Manguifera indica cv Ataulfo) and Papaya (Carica papaya cv Maradol) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) Free Radicals', *PloS one,* 10(11), pp.1-11.

Madureira, A. R., Pereira, A. and Pintado, M. (2016) 'Chitosan nanoparticles loaded with 2,5-dihydroxybenzoic acid and protocatechuic acid: Properties and digestion', *Journal of Food Engineering,* (April 2016)174, pp. 8-14.

Mair, B., Kubicek, S. and Nijman, S. M. B. (2014) 'Exploiting epigenetic vulnerabilities for cancer therapeutics', *Trends in Pharmacological Sciences,* 35(3), pp. 136-145.

Maria, Z., Kaczmarczyk-Sedlak, I., Wojnar, W. and Folwarczna, J. (2018) 'The Effects of Sinapic Acid on the Development of Metabolic Disorders Induced by Estrogen Deficiency in Rats', *Oxidative Medicine and Cellular Longevity,* 2018, pp.1-11.

Markert, T., Moussou, P., Danoux, L. and Rathjens, A. 2011. Use of sinapic acid in non-therapeutic cosmetic and/or topical compositions. Google Patents.

Martirosyan, D. M. and Singh, J. (2015) 'A new definition of functional food by FFC: what makes a new definition unique?', *Functional Foods in Health and Disease,* 5(6), pp. 209-223.

Mathiessen, A. and Conaghan, P. G. (2017) 'Synovitis in osteoarthritis: current understanding with therapeutic implications', *Arthritis Research & Therapy,* 19(18), pp. 1-9.

Michael, L. F., Wu, Z., Cheatham, R. B., Puigserver, P., Adelmant, G., Lehman, J. J., Kelly, D. P. and Spiegelman, B. M. (2001) 'Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1', *Proceedings of the National Academy of Sciences of the U S A,* 98(7), pp. 3820-3825.

Mizrahi, A., Knekt, P., Montonen, J., Laaksonen, M. A., Heliovaara, M. and Jarvinen, R. (2009) 'Plant foods and the risk of cerebrovascular diseases: a potential protection of fruit consumption', *British Journal of Nutrition,* 102(7), pp. 1075-83.

Mohan, R., Jose, S., Mulakkal, J., Karpinsky-Semper, D., Swick, A. G. and Krishnakumar, I. M. (2019) 'Water-soluble polyphenol-rich clove extract lowers pre- and post-prandial blood glucose levels in healthy and prediabetic volunteers: an open label pilot study', *BMC Complementary and Alternative Medicine,* 19(1), pp. 99.

Moors, E. (2012) 'Functional Foods: Regulation and Innovations in the EU', *Innovation The European Journal of Social Science Research,* 25, pp. 424-440.

Naczk, M., Wanasundara, P. K. J. P. D. and Shahidi, F. (1992) 'Facile spectrophotometric quantification method of sinapic acid in hexane-extracted and methanol-ammonia-water-treated mustard and rapeseed meals', *Journal of Agricultural and Food Chemistry,* 40(3), pp. 444-448.

Nithya, R. (2017) 'Sinapic Acid Regulates Glucose Homeostasis by Modulating the Activities of Carbohydrate Metabolizing Enzymes in High Fat Diet Fed-Low Dose STZ Induced Experimental Type 2 Diabetes in Rats', *Global Journal of Obesity, Diabetes and Metabolic Syndrome*, 2017, pp. 054-061.

Nićiforović, N. and Abramovič, H. (2014) 'Sinapic Acid and Its Derivatives: Natural Sources and Bioactivity', *Comprehensive Reviews in Food Science and Food Safety,* 13(1), pp. 34-51.

Nunez-Sanchez, M. A., Gonzalez-Sarrias, A., Romo-Vaquero, M., Garcia-Villalba, R., Selma, M. V., Tomas-Barberan, F. A., Garcia-Conesa, M. T. and Espin, J. C. (2015) 'Dietary phenolics against colorectal cancer--From promising preclinical results to poor translation into clinical trials: Pitfalls and future needs', *Molecular Nutrition and Food Research,* 59(7), pp. 1274-91.

O'Byrne, K. J., Baird, A.-M., Kilmartin, L., Leonard, J., Sacevich, C. and Gray, S. G. (2011) 'Epigenetic regulation of glucose transporters in non-small cell lung cancer', *Cancers,* 3, pp. 1550-1565.

O'Neill, S. and O'Driscoll, L. (2015) 'Metabolic syndrome: a closer look at the growing epidemic and its associated pathologies', *Obesity Reviews,* 16(1), pp. 1-12.

Ogata, A., Kato, Y., Higa, S. and Yoshizaki, K. (2019) 'IL-6 inhibitor for the treatment of rheumatoid arthritis: A comprehensive review', *Modern Rheumatology,* 29(2), pp. 258-267.

World Health Organisation (2019) '*Cardiovascular diseases (CVDs)*'. Available at: https://www.who.int/en/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds) (Accessed: 14/03 2019).

Ozen, A. E., Bibiloni Mdel, M., Pons, A. and Tur, J. A. (2014) 'Consumption of functional foods in Europe; a systematic review', *Nutricion Hospitalaria,* 29(3), pp. 470-8.

Pacurari, M., Kafoury, R., B. Tchounwou, P. and Ndebele, K. (2014) 'The renin-angiotensin-aldosterone system in vascular inflammation and remodelling', *International Journal of Inflammation,* 2014, pp. 1-13.

Padilla-Camberos, E., Lazcano-Díaz, E., Flores-Fernandez, J. M., Owolabi, M. S., Allen, K. and Villanueva-Rodríguez, S. (2014) 'Evaluation of the inhibition of carbohydrate hydrolyzing enzymes, the antioxidant activity, and the polyphenolic content of Citrus limetta peel extract', *The Scientific World Journal,* 2014, pp. 121760-121760.

Pahl, H. L. (1999) 'Activators and target genes of Rel/NF-kappaB transcription factors', *Oncogene,* 18(49), pp. 6853-66.

Poloni, D. M., Dangles, O. and Vinson, J. A. (2019) 'Binding of Plant Polyphenols to Serum Albumin and LDL: Healthy Implications for Heart Disease', *Journal of Agricultural and Food Chemistry,* 67(33), pp. 9139-9147.

Qiu, Y., Liu, Q. and Beta, T. (2010) 'Antioxidant properties of commercial wild rice and analysis of soluble and insoluble phenolic acids', *Food Chemistry,* 121(1), pp. 140-147.

Robbins, R. J. (2003) 'Phenolic acids in foods: an overview of analytical methodology', *Journal of Agricultural and Food Chemistry,* 51(10), pp. 2866-87.

Rodrigues, I. M. M. A., Carvalho, M. G. V. S. and Rocha, J. M. S. (2014) 'Increasing the Protein Content of Rapeseed Meal by Enzymatic Hydrolysis of Carbohydrates', *Bioresources,* 9(2), pp. 2010-2025.

Roy, S. J. and Prince, P. (2012) 'Protective effects of sinapic acid on lysosomal dysfunction in isoproterenol induced myocardial infarcted rats', *Food and Chemical Toxicology,* 50(11), pp. 3984-9.

Russell, R. I. (2001) 'Non-steroidal anti-inflammatory drugs and gastrointestinal damage-problems and solutions', *Postgraduate Medical Journal,* 77(904), pp. 82-88.

Saenglee, S., Jogloy, S., Patanothai, A., Leid, M. and Senawong, T. (2016) 'Cytotoxic effects of peanut phenolics possessing histone deacetylase

inhibitory activity in breast and cervical cancer cell lines', *Pharmacological Reports,* 68(6), pp. 1102-1110.

Sahu, R. P. and Srivastava, S. K. (2011) 'Cruciferous Vegetable-Derived Isothiocyanates and Cancer Prevention', *Functional Foods, Nutraceuticals, and Degenerative Disease Prevention*: Wiley-Blackwell, pp. 147-170.

Saldanha, L. L., Vilegas, W. and Dokkedal, A. L. (2013) 'Characterization of flavonoids and phenolic acids in Myrcia bella Cambess. using FIA-ESI-IT-MS(n) and HPLC-PAD-ESI-IT-MS combined with NMR', *Molecules,* 18(7), pp. 8402-16.

Scazzocchio, B., Vari, R., Filesi, C., Del Gaudio, I., D'Archivio, M., Santangelo, C., Iacovelli, A., Galvano, F., Pluchinotta, F. R., Giovannini, C. and Masella, R. (2015) 'Protocatechuic acid activates key components of insulin signaling pathway mimicking insulin activity', *Molecular Nutrition and Food Research,* 59(8), pp. 1472-81.

Schmitz, M. L. and Baeuerle, P. A. (1991) 'The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B', *Embo Journal,* 10(12), pp. 3805-17.

Sefried, S., Haring, H. U., Weigert, C. and Eckstein, S. S. (2018) 'Suitability of hepatocyte cell lines HepG2, AML12 and THLE-2 for investigation of insulin signalling and hepatokine gene expression', *Open Biology,* 8(10), pp. 1-11.

Senawong, T., Misuna, S., Khaopha, S., Nuchadomrong, S., Sawatsitang, P., Phaosiri, C., Surapaitoon, A. and Sripa, B. (2013) 'Histone deacetylase (HDAC) inhibitory and antiproliferative activities of phenolic-rich extracts derived from the rhizome of Hydnophytum formicarum Jack.: sinapinic acid acts as HDAC inhibitor', *BMC Complementary and Alternative Medicine,* 13(232), pp.1-11.

Sharma, S. (2015) 'Drugs used for the cure of high blood pressure (review)', *International Journal of Scientific Research,* 4(8), pp. 65-66.

Shionoiri, H., Shigemasa, T. and Takasaki, I. (1997) '[Angiotensin-converting enzyme inhibitors: recent therapeutic aspect]', *Nihon Rinsho,* 55(8), pp. 2067-74.

Silambarasan, T., Manivannan, J., Priya, M. K., Suganya, N., Chatterjee, S. and Raja, B. (2015) 'Sinapic acid protects heart against ischemia/reperfusion injury and H9c2 cardiomyoblast cells against oxidative stress', *Biochemical and Biophysical Research Communications,* 456(4), pp. 853-9.

Silambarasan, T., Manivannan, J., Raja, B. and Chatterjee, S. (2016) 'Prevention of cardiac dysfunction, kidney fibrosis and lipid metabolic alterations in l-NAME hypertensive rats by sinapic acid—Role of HMG-CoA reductase', *European Journal of Pharmacology,* 15(777), pp. 113-123.

Singh, U. P., Singh, N. P., Murphy, E. A., Price, R. L., Fayad, R., Nagarkatti, M. and Nagarkatti, P. S. (2016) 'Chemokine and cytokine levels in inflammatory bowel disease patients', *Cytokine,* 77(44-49), pp. 1-12.

Stalikas, C. D. (2007) 'Extraction, separation, and detection methods for phenolic acids and flavonoids', *Journal of Separation Sciences,* 30(18), pp. 3268-95.

Tan, S. H., Mailer, R. J., Blanchard, C. L. and Agboola, S. O. (2011) 'Canola proteins for human consumption: extraction, profile, and functional properties', *Journal of Food Science,* 76(1), pp. R16-28.

Tan, Y., Delvaux, E., Nolz, J., Coleman, P. D., Chen, S. and Mastroeni, D. (2018) 'Upregulation of histone deacetylase 2 in laser capture nigral microglia in Parkinson's disease', *Neurobiology of Aging,* 2018(68), pp. 134-141.

Tortorella, S. M., Royce, S. G., Licciardi, P. V. and Karagiannis, T. C. (2015) 'Dietary Sulforaphane in Cancer Chemoprevention: The Role of Epigenetic Regulation and HDAC Inhibition', *Antioxidants and Redox Signalling,* 22(16), pp. 1382-424.

Tzagoloff, A. (1963) 'Metabolism of Sinapine in Mustard Plants. II. Purification & Some Properties of Sinapine Esterase', *Plant Physiology,* 38(2), pp. 207-13.

Urman, A., Taklalsingh, N., Sorrento, C. and McFarlane, I. M. (2018) 'Inflammation beyond the Joints: Rheumatoid Arthritis and Cardiovascular Disease', *Scifed Journal of Cardiology,* 2(3), pp.1-36.

Velis, C. A. (2015) 'Circular economy and global secondary material supply chains', *Waste Management and Research,* 33(5), pp. 389-391.

Vo, T.-S. and Kim, S.-K. (2013) 'Fucoidans as a natural bioactive ingredient for functional foods', *Journal of Functional Foods,* 5(1), pp. 16-27.

Vuorela, S. (2005) *Analysis, isolation, and bioactivities of rapeseed phenolics.* PhD Dissertation, University of Helsinki.

Vuorela, S., Meyer, A. S. and Heinonen, M. (2003) 'Quantitative analysis of the main phenolics in rapeseed meal and oils processed differently using enzymatic hydrolysis and HPLC', *European Food Research and Technology,* 217(6), pp. 517-523.

Wang, T. and He, C. (2018) 'Pro-inflammatory cytokines: The link between obesity and osteoarthritis', *Cytokine and Growth Factor Reviews,* 44, pp. 38-50.

Welch, C., Zhen, J., Bassene, E., Raskin, I., Simon, J. E. and Wu, Q. (2018) 'Bioactive polyphenols in kinkeliba tea (Combretum micranthum) and their glucose-lowering activities', *Journal of Food and Drug Analysis,* 26(2), pp. 487-496.

Wen, D., Li, C., Di, H., Liao, Y. and Liu, H. (2005) 'A universal HPLC method for the determination of phenolic acids in compound herbal medicines', *Journal of Agricultural and Food Chemistry,* 53(17), pp. 6624-9.

Wilm, M. (2011) 'Principles of electrospray ionization', *Molecular and Cellular Proteomics,* 10(7), pp.1-8.

Wongrakpanich, S., Wongrakpanich, A., Melhado, K. and Rangaswami, J. (2018) 'A Comprehensive Review of Non-Steroidal Anti-Inflammatory Drug Use in The Elderly', *Aging and Disease,* 9(1), pp. 143-150.

Yang, L., Guo, H., Li, Y., Meng, X., Yan, L., Dan, Z., Wu, S., Zhou, H., Peng, L., Xie, Q. and Jin, X. (2016) 'Oleoylethanolamide exerts anti-inflammatory effects on LPS-induced THP-1 cells by enhancing PPARalpha signaling and inhibiting the NF-kappaB and ERK1/2/AP-1/STAT3 pathways', *Scientific Reports,* 6(34611), pp. 1-12.

Yang, Y., Duan, W., Lin, Y., Yi, W., Liang, Z., Yan, J., Wang, N., Deng, C., Zhang, S., Li, Y., Chen, W., Yu, S., Yi, D. and Jin, Z. (2013) 'SIRT1 activation by curcumin pretreatment attenuates mitochondrial oxidative damage induced by myocardial ischemia reperfusion injury', *Free Radical Biology and Medicine,* 65, pp. 667-79.

Yap, A., Nishiumi, S., Yoshida, K.-I. and Ashida, H. (2007) 'Rat L6 myotubes as an in vitro model system to study GLUT4-dependent glucose uptake stimulated by inositol derivatives', *Cytotechnology,* 55(2-3), pp. 103-108.

Yoon, S. and Eom, G. H. (2016) 'HDAC and HDAC Inhibitor: From Cancer to Cardiovascular Diseases', *Chonnam Medical Journal,* 52(1), pp. 1-11.

Yun, K. J., Koh, D. J., Kim, S. H., Park, S. J., Ryu, J. H., Kim, D. G., Lee, J. Y. and Lee, K. T. (2008) 'Anti-inflammatory effects of sinapic acid through the suppression of inducible nitric oxide synthase, cyclooxygase-2, and proinflammatory cytokines expressions via nuclear factor-kappaB inactivation', *Journal of Agricultural and Food Chemistry,* 56(21), pp. 10265-10272.

Zaccardi, F., Webb, D. R., Yates, T. and Davies, M. J. (2016) 'Pathophysiology of type 1 and type 2 diabetes mellitus: a 90-year perspective', *Postgraduate Medical Journal,* 92(1084), pp. 63-69.

Zeier, M. (2018) '[ACE Inhibitors and ARB in Chronic Kidney Disease: What Has to Be Considered]', *Deutsche Medizinische Wochenschrift,* 143(12), pp. 880-885.

Zhang, Q., Hu, J. X., Kui, X., Liu, C., Zhou, H., Jiang, X. and Zeng, L. (2017) 'Sinapic Acid Derivatives as Potential Anti-Inflammatory Agents: Synthesis and Biological Evaluation', *Iranian Journal of Pharmaceutical Research,* 16(4), pp. 1405-1414.

Zhang, X., Shu, X. O., Xiang, Y. B., Yang, G., Li, H., Gao, J., Cai, H., Gao, Y. T. and Zheng, W. (2011) 'Cruciferous vegetable consumption is associated

with a reduced risk of total and cardiovascular disease mortality', *American Journal of Clinical Nutrition,* 94(1), pp. 240-246.

Zheng, S., Deng, S., Huang, Y., Huang, M., Zhao, P., Ma, X., Wen, Y., Wang, Q. and Yang, X. (2018) 'Anti-diabetic activity of a polyphenol-rich extract from Phellinus igniarius in KK-Ay mice with spontaneous type 2 diabetes mellitus', *Food and Function,* 9(1), pp. 614-623.

Zundler, S. and Neurath, M. F. (2015) 'Interleukin-12: Functional activities and implications for disease', *Cytokine and Growth Factor Reviews,* 26(5), pp. 559-68.

Appendix

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Extraction and Quantification of Sinapinic Acid from Irish Rapeseed Meal and Assessment of Angiotensin-I Converting Enzyme (ACE-I) Inhibitory Activity

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ABSTRACT: Phenolic compounds, including phenolic acids, are known to play a protective role against the development of cardiovascular disease. The aim of this work was to generate a phenolic acid extract from Irish rapeseed meal, to determine the quantity of sinapinic acid (SA) in this fraction and to assess the ability of this fraction to inhibit the enzyme angiotensin-I converting enzyme (ACE-I; EC 3.4.15.1). A crude phenolic extract (fraction 1), free phenolic acid containing extract (fraction 2), and an extract containing phenolic acids liberated from esters (fraction 3) were generated from Irish rapeseed meal using a methanol:acetone:water solvent mixture (7:7:6). The total phenolic content (TPC) of each extract was determined and proximate analysis performed to determine the fat, moisture, and protein content of these extracts. Nuclear magnetic resonance (^{1}H NMR) spectroscopy was used to quantify the level of SA in extract 3, which inhibited ACE-I by 91% ± 0.08 when assayed at a concentration of 1 mg/mL, compared to the control, captopril, which inhibited ACE by 97% ± 0.01 when assayed at a concentration of 1 mg/mL.

KEYWORDS: *ACE-I, rapeseed meal, sinapinic acid, solvent extraction, total phenolic content*

1. INTRODUCTION

Rapeseed is one of the world's major oilseeds and a globally valuable crop, with Europe being the largest producer.[1] In 2013/2014, the EU produced 9- 93 million metric tons of rapeseed oil.[2] Rapeseed meal is a byproduct of rapeseed oil production, and this is traditionally used as animal feed. Rapeseed meal is unsuitable for human consumption due to the presence of antinutritional compounds including phytates and glucosinolates, which result in poor digestibility, unpleasant color, and bitter taste.[3] Glucosinolates are hydrolyzed to produce harmful products including oxazolidinethione and isothiocyanate, both of which impair the absorbance of iodine.[4] Erucic acid, or *cis*-13-docosenoic acid, is another antinutritional compound found in high concentrations in rapeseed. The toxic effects of erucic acid are mainly targeted toward the heart, impairing cardiac muscle and altering the fat content of the heart.[4] However, rapeseed is reported to contain more phenolic compounds than any other oilseed plant, with the majority of phenolics remaining in the meal after oil production.[5] These phenolic acids, including sinapinic acid (SA), have gained attention due to their bioactive properties such as antioxidant and antidiabetic activities. SA has also been shown to possess histone deacetylase (HDAC) inhibitory activity, with histone deacetylase inhibitors (HDACi) known to play a protective role against diabetes development.[6] Furthermore, phenolic acids are also thought to play a protective role in heart health and may help to prevent cardiovascular disease (CVD).[7−9]

Clinical studies have shown that the amount of dietary phenolics required to exert their beneficial effects exceeds what is obtainable from dietary intake alone.[10] In this work, phenolic acids containing SA were generated from Irish rapeseed meal using a methanol:acetone:water (7:7:6) solvent system. Cardiovascular disease remains the most common cause of death globally. In 2013 the Global Burden of Disease (GBD) study found that CVD accounted for 17.3 million deaths worldwide.[11] In Europe alone, CVD results in 4 million deaths each year and 45% of all deaths.[11] In terms of CVD, hypertension is a critical factor. ACE-I is a zinc-dependent peptidase which cleaves angiotensin I (a vasodilatory peptide) to angiotensin II, which is a vasoconstrictor peptide involved in regulating blood pressure.[12] Inhibition of ACE-I reduces vasoconstriction and reduces hypertension, therefore improving heart health (Figure 1). The aim of this paper was to generate extracts from Irish rapeseed meal and to assess the impact of these extracts containing SA on the enzyme ACE-I. Given that current ACE-I inhibitors such as captopril and enalapril have various side effects including cough, rash, and vomiting, identifying ACE-I inhibitors from a natural food source is an

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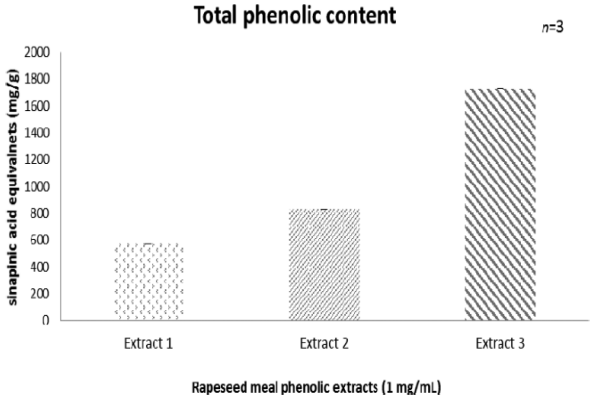

Figure 1. Total phenolic content of Irish rapeseed meal phenolic fractions. All samples were tested at 1 mg/mL. Extract 1 had a TPC of 572 mg SAE/g ± 0.05, with 1832 mg SAE/g ± 0.02 for extract 2 and 1726 mg SAE/g ± 0.08 for extract 3. Data is representative of three biological replicates (n = 3), performed in triplicate. Data is expressed as mean ± SEM, carried out using Excel 2013.

area of interest.[13] This is the first paper to report the ACE-I inhibitory activity of a rapeseed meal phenolic extract containing SA, with potential use as a functional food ingredient.

2. MATERIALS AND METHODS

2.1. Chemicals. Methanol, acetone, captopril, Folin−Ciocalteu phenol reagent, sinapinic acid, and DMSO-d_6 (with 1% TMS v/v) were supplied by Sigma-Aldrich (Dublin, Ireland). The ACE-I kit-WST was supplied by Dojindo Molecular Technologies Inc. Japan.

2.2. Sample Preparation. Rapeseed meal was kindly supplied by Donegal Rapeseed Company, Co. Donegal, Ireland. The rapeseed meal was prepared from seed harvested in the summer of 2014. Rapeseed meal was defatted overnight with hexane using a meal:solvent ratio of 90 g:5 L (w/v). Defatting was carried out at room temperature on a JENWAY 1002 stirrer (Wolflabs, U.K.) and repeated three times. Defatted meal was subsequently air-dried.

2.3. Extraction of Phenolic Acids. Extraction of free phenolic acids and phenolic acids liberated from esters was performed according to the method of Naczk et al. (1992), with modifications as described.[14]

Briefly, phenolic acids were extracted five times from Irish rapeseed using a total meal:solvent ratio of 1:20 (w/v) of methanol:acetone:-water (7:7:6 v:v:v). The extractions were carried out separately with 2 g of meal and 40 mL of methanol:acetone:water (7:7:6 v:v:v) in six separate 50 mL tubes. Extracts were vortexed for 15 s, followed by centrifugation for 15 min at 5000 rpm (2655g) at 20 °C. The supernatants from each extraction were combined and reduced to approximately 240 mL using rotary evaporation at 30 °C. From this, 40 mL was kept, before being dried under nitrogen at 30 °C and labeled as extract 1 (total phenolics). In order to extract the soluble esters, 30 mL of this initial extract was also kept for hydrolysis. The remaining extract was extracted six times with diethyl ether:ethyl acetate (1:1 v:v), evaporated at 30 °C using rotary evaporation, and then was dried under nitrogen at 30 °C. This was labeled as extract 2 (free phenolic acids). To release the esterified phenolic acids, 30 mL of the initial extract was hydrolyzed with 30 mL of 4 M NaOH under nitrogen for 4 h at room temperature, before being acidified to pH 2 with 6 M HCL. The acidified solution was then extracted six times with diethyl ether:ethyl acetate (1:1 v:v) evaporated at 30 °C using rotary evaporation (BUCHI, Switzerland), before being dried under nitrogen at 30 °C. This was labeled as extract 3 (soluble esters).

Multiple extractions were carried out and pooled. From 144 g of defatted rapeseed meal, the yield of extract 1 was 1.003 g (0.7% yield), extract 2 was 0.70 g (0.5% yield), and extract 3 was 2.11 g (1.5% yield). All samples were freeze-dried (Cuddon Engineering, New Zealand) overnight and stored at −20 °C.

2.4. Total Phenolic Content. The total phenolic content was determined using the Folin−Ciocalteu (F−C) assay, according to the method described previously by Khattab et al. (2010).[15] Briefly, the phenolic extracts (1−3) were prepared to a final concentration of 1 mg/mL with methanol, in triplicate (Sigma-Aldrich, Dublin, Ireland). Aliquots (0.2 mL) of each sample were diluted to 0.5 mL with water, and 0.5 mL of Folin−Ciocalteu phenol reagent (Sigma-Aldrich, Dublin, Ireland) was added. After 3 min, 1 mL of 19% sodium carbonate was added to each sample. After 60 min, absorption was measured at 750 nm using a spectrophotometer (Hitachi U-2900). Sinapinic acid (Sigma, Dublin, Ireland) was used as the standard (1−10 mg/mL), and results were expressed as mg of sinapinic acid equivalents per gram (mg SAE/g).

2.5. Protein Analysis. Analysis of protein content was performed on each phenolic extract (1−3) using the Dumas method in accordance with the AOAC Method 968.06 (15th ed.) using a Leco FP629 protein analyzer. A conversion factor of 5.70 was used in order to determine the total protein content from the nitrogen concentration.

2.6. Fat and Moisture Analysis. Total fat was determined for the three phenolic extracts, along with rapeseed meal, defatted rapeseed meal, and rapeseeds. This was carried out using the acid hydrolysis filter bag technique using an automated Ankom Fat Analyzer XT15 based on the method ISO 6492.[16]

2.7. Ash Analysis. The ash content of samples was determined by furnace overnight at 600 °C.[17,18]

2.8. ACE-I Inhibition. Measurement of ACE-I inhibition was performed using the ACE-I kit-WST following the manufacturer's instructions (Dojindo Molecular Technologies Inc., Japan). Briefly, the phenolic extracts (1−3) and positive control (captopril) were assayed in triplicate at a concentration of 1 mg/mL. The concentration of each extract which inhibited ACE-I by 50% (the IC_{50} value) was determined for extract 3 and captopril. Each extract was dissolved in deionized water for the initial assay or 1% DMSO for the IC_{50} determination. Absorbance was measured using an Omega microplate reader at 450 nm, and % inhibition was calculated using the following:

$$[(\text{absorbance blank 1} - \text{absorbance inhibitor}) / (\text{absorbance blank 1} - \text{absorbance blank 2})] \times 100$$

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where blank 1 is the control without the addition of any inhibitor, blank 2 is the reagent blank, and the inhibitor is the positive control or test sample (extracts).

IC_{50} was determined by plotting % ACE-I inhibition against concentration (mg/mL) of extract or captopril, and solving the equation of the line, substituting 50 for the *Y* value and solving for *X*.

2.9. ^{1}H NMR Analysis. ^{1}H NMR spectroscopy was used to quantify the level of sinapinic acid in extracts 1−3. It was performed using the method of Tan and Shahidi (2013), using Topspin software 2.1.[19] A standard curve was prepared using SA (Sigma-Aldrich, Dublin, Ireland), which dissolved in dimethyl sulfoxide-d_6 with 1% (v/v) tetramethylsilane (DMSO-d_6 with 1% TMS (v/v) (Sigma-Aldrich, Dublin, Ireland). TMS served as an internal standard for the analysis. Phenolic extract 3 was analyzed in triplicate by dissolving 0.1 g of sample in 1 mL of DMSO-d_6 containing 1% (v/v) TMS. In order to calculate the quantity of SA in the extract, the average values for each standard were plotted in Excel and a linear curve was generated. The equation of the line was solved, substituting the value obtained for extract 3 for the *Y* value and then solving for *X*.

2.10. Statistical Analysis. Data was derived from samples obtained in biological triplicate, and each experiment was performed in triplicate. The exception was ACE-I IC_{50} determination, which was performed using two biological replicates in triplicate. Data was subsequently analyzed for standard deviation (SD) and standard error of mean (SEM), which was calculated and plotted using Microsoft Excel 2013.

3. RESULTS

3.1. Protein, Fat, Moisture, and Ash Analysis. The moisture, protein, and fat content of phenolic extracts 1−3, along with defatted rapeseed meal and seeds, are shown in Table 1.

Table 1. Protein, Fat, Ash, and Moisture Content of Irish Rapeseed Meal Phenolic Extracts along with Oilseed Rape and Oilseed Rape Meal

sample	protein ($N \times 5.70$)	fat	ash	moisture
extract 3	1.6	4.1	not tested	4.8
oilseed rape	3.1	47.3	4.0	7.3
oilseed rape meal	3.9	25.7	5.3	10.5

3.2. Total Phenolic Content of Phenolic Extracts. The Folin−Ciocalteu assay measures the total phenolic content of samples based on the transfer of electrons in an alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes, forming a blue complex which can be measured using a spectrophotometer.[20] The total phenolic content for each phenolic extract is shown in Figure 2. The total phenolic content ranged from 572 mg SAE/g ± 0.05 for extract 1 to 1832 mg SAE/g ± 0.02 for extract 2 and 1726 mg SAE/g ± 0.08 for extract 3.

3.3. Rapeseed Phenolic Extracts Inhibit ACE-I Activity. Extract 3 was examined for its potential to inhibit the enzyme ACE-I, with ACE-I known to play a role in heart health by ameliorating hypertension. The ACE-I inhibitory values obtained for the phenolic extracts 1−3 are shown in Figure 3. ACE-I inhibitory activity ranged from 89% ± 0.04 for extract 1 to 90% ± 0.01 for extract 2 and 91% ± 0.08 for extract 3. The ACE-I inhibitory activity values of the three extracts were comparable to those of the positive control, captopril (97% ± 0.01), at a concentration of 1 mg/mL.

3.3.1. ACE-I IC_{50} Values. The concentration of extract 3 that inhibited ACE-I by 50% (the IC_{50} value) was determined and compared to the positive control, captopril. Extract 3 was chosen for further analysis as it inhibited ACE-I by 91% ± 0.08. Extract 3 had an IC_{50} value of 0.25 mg/mL ± 0.05, compared to the positive control, captopril, which had an IC_{50} value of 0.05 mg/mL ± 0.01, which is lower than reported in the literature, at approximately 0.004 mg/mL.[21]

3.4. ^{1}H NMR Analysis. ^{1}H NMR spectroscopy was performed in order to quantify the level of SA in extract 3, given that it displayed the highest total phenolic content and ACE-I inhibitory activity (Figures 4 and 5). Using the equation of the line generated from the standard curve of SA, extract 3 was found to contain 5.31 mg of SA (5.3%) per 100 mg of extract.

DISCUSSION

Rapeseed meal is a low economic value byproduct of rapeseed oil production and is ordinarily used as animal feed. Rapeseed contains more phenolic compounds than any other oilseed plant, and the majority of these phenolics remain in the meal after oil production. Phenolic acids including SA are also found in fruits, vegetables, and members of the Brassicaceae family including broccoli, cabbage, and kale. However, isolating these potentially valuable phenolic acids from rapeseed meal could enhance the economic value of this resource and make use of a byproduct which is unfit for human consumption. It also fits in with the concept of a "circular economy", whereby resources which would otherwise be considered waste are recycled back into the economy.[22]

A review of the literature regarding the phenolic composition of rapeseed meal identified that SA comprises between 73 and 85.4% of free phenolic acids.[23,24] Up to 80% of phenolic acids in rapeseed meal are esterified, and hydrolysis with sodium hydroxide releases the esterified phenolic acids, including sinapine, yielding SA as the main phenolic acid.[23] According to the literature, SA constitutes between 70.9 and 96.7% of the soluble esters in rapeseed; therefore extracts were successfully generated containing free phenolics and soluble esters using the method of Naczk et al.[14,23] This method was chosen as it is a published method which allows the extraction of phenolic acids in their different forms, i.e., free or esterified, using methanol:acetone:water (7:7:6). During the extraction process, all solvents are completely evaporated, leaving our extract of interest for use in downstream studies. Extract 3, containing the soluble esters, was found to have the highest yield at 2.11 g (1.5% yield). It has previously been shown that esterified phenolics appear to be extracted more effectively than free phenolic acids, as is the case in this work.[14] However, no explanation has been proposed for this observation. Analysis of the total phenolic content found that extract 3 contained the highest level of total phenolics at 1726 mg SAE/g. Given that extract 3 had the highest total phenolic content, ^{1}H NMR spectroscopy was used to determine the quantity of SA in this extract. Quantitative analysis of extract 3 using ^{1}H NMR analysis found that extract 3 contained 5.31 mg/100 mg of extract. This is higher than the values previously reported, which ranged from 0.17−1.7 mg/g of SA in rapeseed meal phenolic isolates.[25] The high level of SA in extract 3 is likely a result of multiple extractions which were carried out to generate a sufficient quantity of the extract. Efforts will be made in order to upscale the extraction of extract 3 from rapeseed meal, using techniques such as accelerated solvent extraction (ASE).

All three extracts generated in this work were found to inhibit ACE-I, with extract 3 found to inhibit ACE-I by 91% ± 0.08. ACE-I inhibition has also been reported for other phenolic

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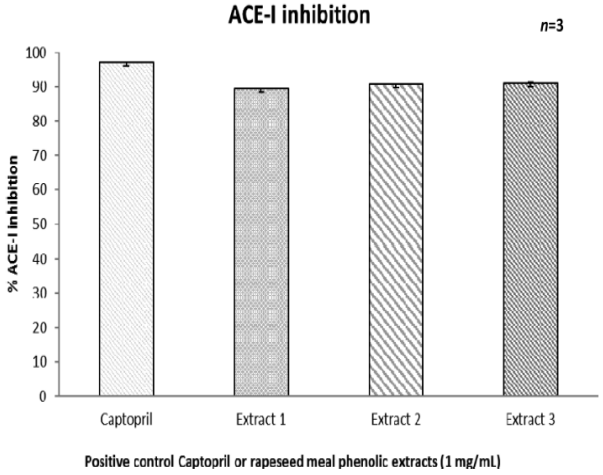

Figure 2. Angiotensin I-converting enzyme inhibition by Irish rapeseed meal phenolic fractions and positive control captopril. All samples were tested at 1 mg/mL. ACE-I inhibitory activity ranged from 89% ± 0.04 for extract 1 to 90% ± 0.01 for extract 2 and 91% ± 0.08 for extract 3. Data is representative of three biological replicates (n = 3), performed in triplicate. Data is expressed as mean ± SEM, carried out using Excel 2013.

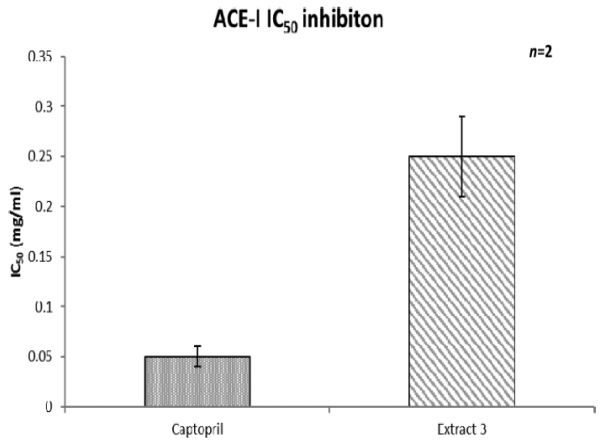

Figure 3. Angiotensin-I converting enzyme IC_{50} inhibition values for positive control, captopril, and phenolic fraction 3. Extract 3 had an IC_{50} value of 0.25 mg/mL ± 0.05, compared to the positive control, captopril, which had an IC_{50} value of 0.05 mg/mL ± 0.01. Data is representative of 2 independent experiments (n = 2), performed in triplicate. Data is expressed as mean ± SEM, carried out using Excel 2103.

extracts generated from fruits and vegetables. For example, phenolic extracts from black and red raspberries were found to inhibit ACE-I by over 70% when assayed previously.[26] ACE-I is a zinc-dependent peptidase which cleaves angiotensin I to angiotensin II, which is a vasoconstrictor involved in regulating blood pressure.[12] Recent evidence suggests that various processes including oxidative stress, inflammation, and hypertension are interconnected, with each influencing the other in terms of the pathology of "hypertension-related cardiovascular events".[27] Therefore, developing inhibitors which can target ACE-I and which also possess antioxidant activity is of great interest.[27] The antioxidant activity of phenolic acids including sinapinic acid is well-known.[7,28] The inflammatory process is also known to contribute to cardiovascular events related to hypertension, and it has been shown that ACE-I inhibitors can alter inflammatory molecules.[29] Such molecules include intercellular adhesion-molecule-1, tumor necrosis factor-α, and C-reactive protein.[29] In line with this, SA has been shown to exert its anti-inflammatory activity by inhibiting the expression of iNOS, COX-2, TNF-α, and IL-1β.[30] SA could potentially play a role in the treatment of not only hypertension but also disease states caused by hypertension; these include vascular hypertrophy, retinopathy, and stroke.[31]

Inhibition of ACE-I is just one potential mechanism by which SA could potentially improve heart health. SA has been shown to restore ventricular function and improve both percent rate pressure product and percent coronary flow.[7,9] Therefore, further studies are necessary to examine the effects of SA through confocal analysis of stress fibers and focal adhesions.

A potential mechanism has been proposed by which phenolic acids, including hydroxycinnamic acids like SA, inhibit ACE-I.[32] The above study found that hydroxycinnamic acids had

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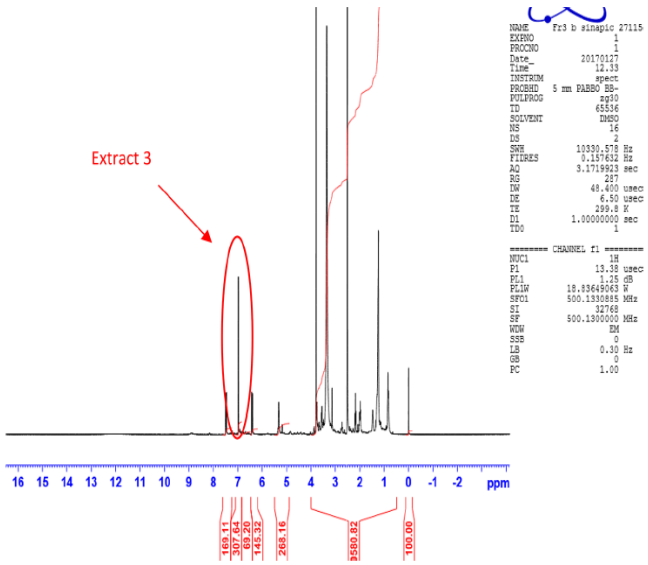

Figure 4. [1]H NMR spectra of extract 3. The peak for extract 3 is highlighted by the red circle. Extract 3 was analyzed in triplicate.

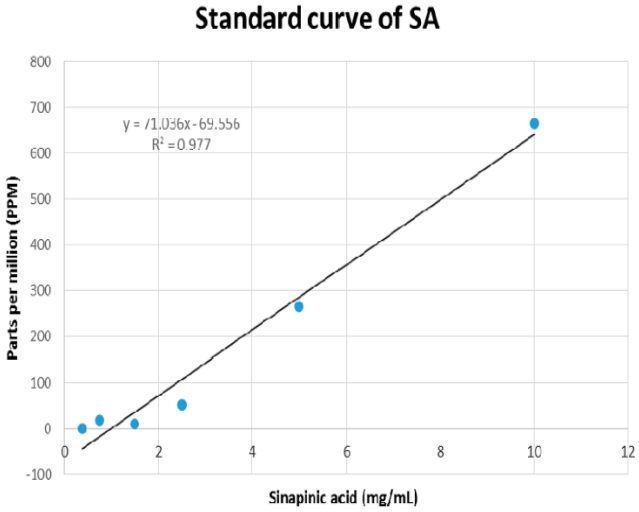

Figure 5. Standard curve of SA. The data generated from [1]H NMR analysis of SA at various concentrations was used to generate a standard curve. The equation of the line and *R*-squared value are displayed, which were used to calculate the mg of SA in extract 3.

increasing ACE-I inhibitory activity with increasing numbers of hydroxyl groups. They also found that the potency of ACE-I inhibition depends upon the presence of particular functional groups, including hydroxyl, carboxyl, and acrylic groups, which can act as hydrogen-bond acceptors or donors.[32] Gallic acid, which possesses five hydrogen-bond acceptor groups just as SA does, was found to have higher activity when compared to syringic acid, which has a low number of hydrogen-bond acceptor groups.[32]

The ACE-I inhibitory properties of SA mean that it could also prove potentially useful in the treatment of other diseases including cancer, with a recent study demonstrating improved survival in adenocarcinoma patients following treatment with ACE-I inhibitors.[33]

The fat content determined for the oilseed rape sample was higher than that obtained for the oilseed meal. This would be expected, as during the pressing of the oilseed rape to produce rapeseed oil, much of the oil and lipid content is expelled. Oilseed rape meal is a byproduct of this deoiling process, and therefore is expected to contain less fat than the oilseed rape itself (Table 1). The ash content of rapeseed has been reported to be approximately 4%, with our oilseed rape found to have an ash content of 4.01%. Values reported for rapeseed meal have been reported to be slightly higher at around 8%, with similar

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values obtained with our samples, as the oilseed meal had a slightly higher ash content at 5.3%.[4]

However, there are limitations to this work. The phenolic extract isolated as part of this work is not purified and, therefore, contains other phenolic acids besides SA. Some of these include protocatechuic, caffeic, and syringic acid.[14] PCA was found to be a poor inhibitor of ACE-I activity, with less than 50% inhibition observed at a concentration of 0.5 mM.[34] Another study found that, at a similar concentration of 0.8 mM, PCA only displayed 9% ACE inhibition.[32] When the concentration was increased to 10 mM, the ACE-I inhibitory activity increased to 91%.[32] Syringic acid has also been reported to be a weak inhibitor of ACE-I, with 54% inhibition at a concentration of 10 mM.[32] Similarly, caffeic acid has been shown to have poor ACE-I inhibitory activity, displaying just 34% ACE-I inhibition at a concentration of 0.93 mM. In the same study, caffeic acid was unable to be studied at a higher molarity due to its poor solubility.[32] Based on the existing evidence, it appears unlikely that these phenolics contribute to the ACE-I inhibitory effects of our generated extract. Given the high concentration of SA in our generated extract, 5.3 mg or 23 mM, and that a previous study has shown that SA can lower plasma ACE activity, it would appear that SA does contribute to the observed ACE-I inhibitory activity of our extract.[9]

The yield of extract generated was low, and as such multiple extractions were required. Given that the extract displayed good ACE-I inhibitory activity, it is a promising candidate to move forward into animal studies, to determine its efficacy as an antihypertensive agent in vivo, but also for use in toxicology studies. As such, it is planned to upscale the extraction process and move forward to in vivo studies as mentioned. Another limitation of this work is the stability of the extract, which is yet to be determined. This information would be pertinent regarding the potential use of this extract as a functional food ingredient, which is ultimately what the project aims to achieve.

In conclusion, a phenolic extract isolated from Irish rapeseed meal has potential utility as an antihypertensive agent to improve heart health, through inhibition of ACE-I and decreased hypertension. Therefore, this phenolic extract containing SA is a promising candidate to move forward to in vivo studies for the treatment of hypertension-related cardiovascular events.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ACE-I, angiotensin I-converting enzyme; ASE, accelerated solvent extraction; CVD, cardiovascular disease; F−C, Folin−Ciocalteu; GBD, global burden of disease; NMR, nuclear magnetic resonance; SA, sinapinic acid; TMS, tetramethylsilane; TPC, total phenolic content

REFERENCES

(1) Carre, P.; Pouzet, A. *OCL: Oilseeds Fats, Crops Lipids* **2014**, *21*, 1−12.
(2) List, G. *Lipid Technol.* **2015**, *27*, 120−120.
(3) Tan, S. H.; Mailer, R. J.; Blanchard, C. L.; Agboola, S. O. *J. Food Sci.* **2011**, *76*, R16−28.
(4) Dingyuan, F.; Jianju, Z. *Rapeseed Conference, 12th IRC* (Wuhan, China); 2007; pp 265−270.
(5) Vuorela, S. *Analysis, isolation, and bioactivities of rapeseed phenolics*, PhD Thesis, University of Helsinki, 2005; pp 1−75.
(6) Senawong, T.; Misuna, S.; Khaopha, S.; Nuchadomrong, S.; Sawatsitang, P.; Phaosiri, C.; Surapaitoon, A.; Sripa, B. *BMC Complementary Altern. Med.* **2013**, *13*, 232. Raichur, S.; Teh, S. H.; Ohwaki, K.; Gaur, V.; Long, Y. C.; Hargreaves, M.; McGee, S. L.; Kusunoki, J. *J. Mol. Endocrinol.* **2012**, *49*, 203−211.
(7) Silambarasan, T.; Manivannan, J.; Priya, M. K.; Suganya, N.; Chatterjee, S.; Raja, B. *Biochem. Biophys. Res. Commun.* **2015**, *456*, 853−859.
(8) Cherng, Y. G.; Tsai, C. C.; Chung, H. H.; Lai, Y. W.; Kuo, S. C.; Cheng, J. T. *J. Agric. Food Chem.* **2013**, *61*, 12053−12059.
(9) Silambarasan, T.; Manivannan, J.; Raja, B.; Chatterjee, S. *Eur. J. Pharmacol.* **2016**, *777*, 113−123.
(10) Nunez-Sanchez, M. A.; Gonzalez-Sarrias, A.; Romo-Vaquero, M.; Garcia-Villalba, R.; Selma, M. V.; Tomas-Barberan, F. A.; Garcia-Conesa, M. T.; Espin, J. C. *Mol. Nutr. Food Res.* **2015**, *59*, 1274−1291.
(11) Townsend, N.; Wilson, L.; Bhatnagar, P.; Wickramasinghe, K.; Rayner, M.; Nichols, M. *Eur. Heart J.* **2016**.
(12) Bernstein, K. E.; Ong, F. S.; Blackwell, W. L.; Shah, K. H.; Giani, J. F.; Gonzalez-Villalobos, R. A.; Shen, X. Z.; Fuchs, S. *Pharmacol. Rev.* **2013**, *65*, 1−46.
(13) Sharma, S. *Int. J. Sci. Res.* **2015**, *4*, 65−66.
(14) Naczk, M.; Wanasundara, P. K. J. P. D.; Shahidi, F. *J. Agric. Food Chem.* **1992**, *40*, 444−448.
(15) Khattab, R.; Eskin, M.; Aliani, M.; Thiyam, U. *J. Am. Oil Chem. Soc.* **2010**, *87*, 147−155.
(16) Seenger, J.; Nuernberg, G.; Hartung, M.; Szucs, E.; Ender, K.; Nuernberg, K. *Arch Tierz., Dummerstorf* **2008**, *51*, 449−457.
(17) Arif, M.; Nasiruddin; Masood, T.; Shah, S. S.; Faayaz, H. *ARPN J. Agric. Biol. Sci.* **2012**, *7* (9).
(18) AOAC (Association of Official Analytical Chemists). Helrich, K., Ed.; AOAC: Arlington, VA, 1990.
(19) Tan, Z.; Shahidi, F. *Food Chem.* **2013**, *138*, 1438−1447.
(20) Ainsworth, E. A.; Gillespie, K. M. *Nat. Protoc.* **2007**, *2*, 875−877.
(21) Ben Henda, Y.; Labidi, A.; Arnaudin, I.; Bridiau, N.; Delatouche, R.; Maugard, T.; Piot, J. M.; Sannier, F.; Thiery, V.; Bordenave-Juchereau, S. *J. Agric. Food Chem.* **2013**, *61*, 10685−10690.
(22) Velis, C. A. *Waste Manage. Res.* **2015**, *33*, 389−391.
(23) Shahidi, F.; Naczk, M. *J. Am. Oil Chem. Soc.* **1992**, *69*, 917−924.
(24) Thiyam, U.; Stöckmann, H.; Zum Felde, T.; Schwarz, K. *Eur. J. Lipid Sci. Technol.* **2006**, *108*, 239−248.
(25) Vuorela, S.; Meyer, A. S.; Heinonen, M. *J. Agric. Food Chem.* **2004**, *52*, 8202−8207. Nićiforović, N.; Abramovič, H. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 34−51.
(26) Cheplick, S.; Kwon, Y.-I.; Bhowmik, P.; Shetty, K. *J. Food Biochem.* **2007**, *31*, 656−679.
(27) Pacurari, M.; Kafoury, R.; Tchounwou, P. B.; Ndebele, K. *Int. J. Inflammation* **2014**, *2014*, 1−13.
(28) Gaspar, A.; Martins, M.; Silva, P.; Garrido, E. M.; Garrido, J.; Firuzi, O.; Miri, R.; Saso, L.; Borges, F. *J. Agric. Food Chem.* **2010**, *58*, 11273−11280.
(29) Ferrario, C. M.; Strawn, W. B. *Am. J. Cardiol.* **2006**, *98*, 121−128.
(30) Yun, K. J.; Koh, D. J.; Kim, S. H.; Park, S. J.; Ryu, J. H.; Kim, D. G.; Lee, J. Y.; Lee, K. T. *J. Agric. Food Chem.* **2008**, *56*, 10265−10272.
(31) Harlan, S. M.; Ostroski, R. A.; Coskun, T.; Yantis, L. D.; Breyer, M. D.; Heuer, J. G. *Am. J. Physiol Regul Integr Comp Physiol* **2015**, *309*, R467−474.

DOI: 10.1021/acs.jafc.7b02670
J. Agric. Food Chem. 2017, 65, 6886−6892

(32) Al Shukor, N.; Van Camp, J.; Gonzales, G. B.; Staljanssens, D.; Struijs, K.; Zotti, M. J.; Raes, K.; Smagghe, G. *J. Agric. Food Chem.* **2013**, *61*, 11832−11839.
(33) Al-Shamsi, H. O.; Shalaby, A.; Dar, A. Y.; Hassan, M.; Wolff, R. A.; Li, D. *J. Clin. Oncol.* **2016**, *34*, 420.
(34) Hidalgo, M.; Martin-Santamaria, S.; Recio, I.; Sanchez-Moreno, C.; de Pascual-Teresa, B.; Rimbach, G.; de Pascual-Teresa, S. *Genes Nutr.* **2012**, *7*, 295−306.

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Sinapinic and protocatechuic acids found in rapeseed: isolation, characterisation and potential benefits for human health as functional food ingredients

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Abstract

Rapeseed is one of the world's major oilseeds, and rapeseed oil is produced by pressing of the seeds. This process results in the production of a low-economic-value by-product, rapeseed meal, which is commonly used as animal feed. Rapeseed meal is rich in bioactive phenolic compounds, including sinapinic acid (SA) and protocatechuic acid (PCA). Isolation of these bioactive compounds from a by-product of rapeseed oil production is largely in agreement with the current concept of the circular economy and total utilisation of crop harvest using a biorefinery approach. In this review, current information concerning traditional and novel methods to isolate phenolic compounds – including SA and PCA – from rapeseed meal, along with in vitro *and* in vivo *studies concerning the bioactivity of SA and PCA and their associated health effects, is collated. These health effects include anti-inflammatory, anti-cancer, anti-diabetes activities, along with histone deacetylase inhibition and protective cardiovascular, neurological and hepatic effects. The traditional extraction methods include use of solvents and/or enzymes. However, a need for simpler, more efficient methodologies has led to the development of novel extraction processes, including microwave-assisted, ultrasound-assisted, pulsed electric field and high-voltage electrical discharge extraction processes.*

Keywords

cardiovascular health • diabetes • protocatechuic and sinapinic acid

Introduction

Rapeseed is a globally valuable crop, and its production has steadily grown over the past 20 yr. Rapeseed is cultivated in China, India, Canada and Europe; however, in terms of rapeseed production, Europe is the largest producer (Carre, 2014). In the year 2013/2014, the European Union (EU) produced 9.93 million t of rapeseed oil, while China produced 6.58 million t (List, 2015). A by-product of the rapeseed oil industry, rapeseed meal contains bioactive constituents, such as the phenolics sinapinic acid (SA) and protocatechuic acid (PCA), which have potential uses as functional food ingredients and for use in cosmetic and pharmaceutical applications. Improving and developing methods to isolate phenolic compounds from rapeseed meal could enhance the economic value of this resource. The isolation of phenolics from rapeseed meal encompasses the idea of a "circular economy", whereby resources that would otherwise be considered waste are recycled into products (Velis, 2015). Traditional methods of isolating phenolics from rapeseed meal include organic solvent and enzymatic extraction; however, this can require large volumes of solvent, while the associated toxicity of certain organic solvents such as methanol makes them unsuitable for use in final food applications. This has encouraged the development of novel isolation methods such as ultrasound-assisted (UAE), microwave-assisted (MAE), high-voltage electrical discharge (HVED) and pulsed electric field (PEF) extraction processes (Teh and Birch, 2014; Barba *et al.*, 2015; Teh *et al.*, 2015). In the first part of this review, existing and novel methods of isolating these phenolic compounds from rapeseed meal are examined. The second part of the review focusses on the diverse bioactive properties of these phenolics, which could make them potentially useful in improving human health.

Poor diet plays a role in the development of major diseases such as heart disease, diabetes and cancer. Consumption of a diet rich in vegetables is strongly implicated as a protective factor against such diseases, due to the presence of polyphenols or phenolic compounds. Clinical trials regarding the use of dietary phenolic compounds for the prevention of colorectal cancer

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revealed that the amount required to exert their effects exceeds what is obtainable from dietary intake alone (Nunez-Sanchez *et al.*, 2015). In this review, diverse and potentially important physiological roles of SA and PCA in terms of human health and disease are discussed.

Rapeseed meal and oil production

Rapeseed meal is a by-product of the rapeseed de-oiling process, with the majority of phenolic compounds remaining in the meal after extraction of the oil (Figure 1). Traditionally, the production of rapeseed oil involves mechanical extraction by pre-pressing and extrusion of rapeseeds, followed by extraction with solvents such as food grade hexane. The processing of oil and meal involves various steps, including seed pre-conditioning and flaking, seed cooking, pressing and solvent extraction, followed by the removal of solvent and toasting. Some small-scale facilities do not use solvents to extract rapeseed oil, using only mechanical extraction (Booth, 2004). Although food grade solvents are used during extraction of food grade oils, further refinement of the rapeseed oil is necessary to make it more palatable and suitable for human consumption. Refinement steps include the removal of unwanted components, including phospholipids and free fatty acids, which can be achieved by either physical or chemical means. Chemical methods include either water or acid degumming and alkali refining, while the physical process involves "steam distillation".

Phenolic compounds in rapeseed

Rapeseed is reported to contain more phenolic compounds than any other oilseed plant (Lomascolo *et al.*, 2012). Reports suggest that rapeseed contains 240–590 µg/g of SA, while rapeseed cake (an initial by-product produced by crushing the rapeseeds to remove the oil) contains 170–454 µg/g of SA (Nićiforović and Abramovič, 2014). PCA is also found in rapeseed, with concentrations ranging from 28 µg/g to 612 µg/g in rapeseed hulls (Liu *et al.*, 2012). Phenolic compounds present in rapeseed can be categorised into various subgroups: phenolic acids, lignans, stilbenes, flavonoids, isoflavonoids and complex phenolic polymers such as tannins (Vuorela, 2005). Phenolic acids tend to be found in a soluble form, conjugated with sugars or organic acids, and are usually constituents of more complex structures such as lignans and hydrolysable tannins (Cheynier *et al.*, 2013). In rapeseed, the main phenolic compounds are derivatives of hydroxycinnamic acid (Cvjetko. *et al.*, 2009). The biosynthetic origin of both benzoic and cinnamic acid derivatives is the aromatic amino acid L-phenylalanine, which itself is the end product of the shikimate pathway. The preceding conversion of L-phenylalanine to various hydroxycinnamic acids involves a three-step sequence, referred to as the "general phenylpropanoid metabolism" (Robbins, 2003). Phenolic compounds found in rapeseed mainly exist in the esterified form. Sinapine, which is the choline ester of SA, is the main phenolic ester in rapeseed.

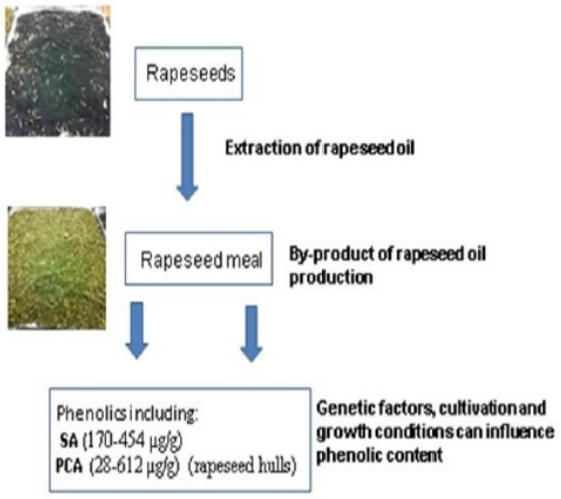

Figure 1. Rapeseed de-oiling process and the phenolics remaining in the meal after extraction.

However, SA can also be esterified to other phenolic acids, sugars or kaempferols (Vuorela, 2005). SA comprises 70%–96% of the esterified phenolic acids in rapeseed meal, while PCA is present in small quantities in rapeseed flours (Shahidi and Naczk, 1992). Sinapine plays an important role in rapeseed, acting as storage for both SA and choline in young plants (Kozlowska *et al.*, 1990). As the seeds mature, some of the sinapine is enzymatically hydrolysed by sinapine esterase to choline and free SA (Tzagoloff, 1963). Apart from the esterified forms, free phenolic acids can also be found in rapeseed, comprising up to 15% of the total phenolics in rapeseed meal. SA comprises 65%–85% of free phenolic acids in defatted rapeseed meals, with PCA also present in small quantities (Naczk *et al.*, 1992; Shahidi and Naczk, 1992; Vuorela, 2005).

Sources and derivatives of SA

SA is found in varying amounts primarily among citrus fruits. For example, lemon and Murcott orange are known to contain the highest quantities of SA in comparison to other natural sources. Additionally, cereal grains such as rye and rice are also known to contain substantial concentrations of SA, while it has also been shown to be present in various herbs such as sage and thyme. SA and its derivatives are particularly abundant among the different varieties of *Brassica* vegetables, including rapeseed, kale, white cabbage, turnip and broccoli (Table 1). In terms of its structure, SA is 3, 5-dimethoxy-4-hydroxycinnamic acid, which is found either in free or esterified form. The most common glycoside of SA is 1-*O*-β-d-glucopyranosyl sinapate, which can be found in numerous Brassicaceae species. SA can also form dimers with itself and ferulic acid in the walls of cereal cells, with C8–C8-coupled SA dehydrodimers and three sinapate-ferulate heterodimers discovered in various insoluble and soluble cereal grain dietary fibres. SA in rapeseed also exists as the glucosidic ester, glucopyranosyl sinapate. SA does not usually exist in free form, and <16% of SA is present as free SA. Moreover, 4-vinylsyringol, which is found in crude rapeseed oil, is a decarboxylation product of SA. During rapeseed oil extraction, elevated temperatures and pressures cause SA to undergo structural changes, resulting in the formation of 4-vinylsyringol, a decarboxylation product of SA, along with syringaldehyde. The amount of SA derivatives (which include sinapoyl glucose, methyl sinapate, sinapoyl malate and 1-sinapoyl-2-feruloylgentiobiose) found in rapeseed ranges between 6,390 and 18,370 µg/g, but this is dependent on the method used to process the oil (Lomascolo *et al.*, 2012; Nićiforović and Abramovič, 2014).

Sources and structure of PCA

PCA is chemically known as 3,4-dihydroxybenzoic acid. This compound is purported to have various pharmacological activities, including anti-inflammatory, anti-cancer and cardiac beneficial activity. It is frequently detected in edible plants used in folk medicine and is one of the suggested biologically active components in medicinal plants such as Sudan Mallow (*Hibiscus sabdariffa L.*) and St. John's wort (*Hypericum perforatum L.*) (Kakkar and Bais, 2014). It is also found in common fruits and vegetables, such as blackberries, blackcurrant, cauliflower and lentils (Masella *et al.*, 2012). PCA is a major metabolite of other complex polyphenols, such as the anthocyanins. Anthocyanins are mainly found in fruits such as those of the berry family, pomegranate and pigmented varieties of oranges (e.g. Tarocco, Moro and Sanguinello). In humans and animals, anthocyanins are absorbed as intact glycosides and metabolites. While they can undergo methylation and glucuronidation, they primarily undergo degradation, followed by considerable phase II conjugation, particularly methylation and sulphation. De Ferrars *et al.* (2014) studied the metabolism of the anthocyanin cyanidin-3-glucoside and found that it spontaneously degrades to PCA in the small intestine and the circulation (De Ferrars *et al.*, 2014).

Traditional extraction processes for recovering phenolics from rapeseed meal

Traditionally, extraction of phenolic acids involves a de-fatting step to remove excess oil. This is usually performed with hexane in a Soxhlet apparatus, with the use of food grade hexane important if the product is to be utilised in food applications. Phenolics found in rapeseed can be divided into three categories: (1) free phenolic acids, (2) soluble esters and (3) insoluble-bound phenolics (Naczk *et al.*, 1992; Szydłowska-Czerniak *et al.*, 2010). Solvents used to extract these phenolics include methanol, ethanol, acetone or a combination of these (e.g. methanol: acetone: water 7:7:6) (Naczk *et al.*, 1992; Cai and Arntfield, 2001; Khattab *et al.*, 2010; Liu *et al.*, 2012). Free phenolic acids can be extracted using methanol: acetone: water (7:7:6, v:v:v) solvent system, and the soluble esters can be obtained through hydrolysis with sodium hydroxide or enzymes and subsequently extracted with diethyl ether: ethyl acetate (1:1, v:v) (Naczk *et al.*, 1992). Extraction and hydrolysis can also be performed with enzymes, such as Ultraflo L and ferulic acid esterase (Vuorela *et al.*, 2003). The main compound released using enzymatic extraction is SA, with a study by

Vuorela (2005) revealing that enzymatic extractions yielded a higher total phenolic content compared to solvent-based extractions. Enzymes are also useful agents for isolation in that they are considered food-friendly, which makes the isolated compounds suitable for food applications (Vuorela, 2005).

Limitations of solvent extraction

Given the shift towards "green and sustainable" methods to extract bioactive compounds, reducing or eliminating solvent use is a key factor when developing new methodologies. This is due to the harmful properties of organic solvents; they are flammable, volatile and toxic, while they may also cause environmental pollution (Chemat *et al.*, 2012). The use of organic solvents also poses problems in terms of using the extracted compounds in food applications. In order for SA and PCA to be used as food components, the solvents used to extract them must be food grade and have safe status. If solvents with toxic properties are used in the extraction process, they must be removed to a specified residual level in order to be eligible for use in food applications, e.g. 5–30 mg of hexane/kg of product (such as edible oils) (Starmans, 1996).

Novel extraction processes for recovering phenolics from rapeseed meal

Developing novel methods to isolate phenolic compounds from rapeseed meal not only overcomes the issues associated with the use of solvents but also closely links with the biorefinery concept. Efficient, simpler and more economic methods of isolation allow for the utilisation of rapeseed by-products and ensure total utilisation of the crop, with no or limited waste generation (Chemat *et al.*, 2012). Various novel methods to extract phenolic acids from various natural product sources have been carried out to date, including UAE, MAE, HVED and PEF extractions (Teh and Birch, 2014; Barba *et al.*, 2015; Teh *et al.*, 2015).

UAE process

UAE provides a unique advantage over traditional methods of extraction in that it can overcome physical barriers, such as poor solubility of phenolics in rapeseed cake, which can make extraction difficult. These physical barriers arise as phenolics in the cake are encompassed in lignin, hulls and cell walls, which are insoluble structures. This, along with the fact that phenolic acids, including SA, occur as esters, glycosides and insoluble-bound forms, has hindered their extraction from rapeseed cake. Ultrasound generates sound waves that create "cavitation bubbles" close to the sample tissue; this leads to the breakdown and disruption of cell walls and release of the bioactive contents (Khoddami *et al.*, 2013). Teh and Birch (2014) previously determined the optimal parameters to extract phenolics from defatted canola seed cakes to comprise a 200 W ultrasound extraction with a solvent volume of 50 mL, extraction time of 20 min, and temperature of 70°C. This method was found to be advantageous over traditional methods in that it involves low cost, has low solvent consumption and increases the antioxidant activity of the extracts (Teh and Birch, 2014). Other protocols have demonstrated UAE to be a suitable technique to extract phenolics from rapeseed and related species. An optimised protocol for the UAE of antioxidant compounds, including phenolics, from rapeseed cultivars was previously described by Szydłowska-Czerniak and Tułodziecka (2014). They concluded that the optimal conditions to perform UAE were an extraction time of 13.8 min, a solvent-to-material ratio of 26.2 mL/g and a solvent concentration of 50.3% (Szydłowska-Czerniak and Tułodziecka, 2014). Another study investigated the optimal UAE conditions to extract phenolics from kale, which is a member of the Brassicaceae family, along with rapeseed. They determined that the optimal conditions were 80% ethanol, with an extraction time of 60 min, ultrasound frequency of 20 kHz and power of 100 W (Oniszczuk and Olech, 2016). Dubie *et al.* (2013) also extracted phenolic antioxidant compounds from mustard (*Brassica juncea*) seed meal, another member of the Brassicaceae family, using UAE. They determined that the optimal conditions were 75°C for 20 min with 70% ethanol and a solvent-to-meal ratio of 40:1, with 20 kHz and 0.5 W/mL ultrasound treatments using a Branson Sonifier probe with input power of 400 W (Dubie *et al.*, 2013).

MAE process

MAE of phenolics from rapeseed meal offers advantages, including shorter extraction times and greater yields, over traditional methods such as solvent extraction. Teh *et al.* (2015) investigated MAE of phenolics from rapeseed cakes. The optimal parameters for MAE of rapeseed cakes were found to be 5 min, with a liquid: solvent ratio of 6 mL/g and power of 633.33 W. Microwave pre-treatment using the optimal parameters described maximised the phenolic yield from rapeseed cake when subsequently extracted with ultrasound and methanol: acetone: water mixture (7:7:6, v:v:v). This method was economically viable due to the shorter extraction time, moderate microwave power and low electroporation voltage and frequency (Teh *et al.*, 2015). MAE has also been used in the extraction of phenolic compounds from broccoli, which is a member of the Brassicaceae (or mustard) family along with rapeseed. The optimal conditions were determined to be 71.11°C, microwave power of 167.03 W, solvent concentration (methanol/water) of 75.95% and an extraction time of 16.34 min (Jokić *et al.*, 2012). Other

studies have demonstrated the efficacy of this technique in the extraction of phenolics, although from different sources, including rice grains and almond skin by-products (Setyaningsih *et al.*, 2015; Valdés *et al.*, 2015). The success of this technique in extracting phenolic compounds means it could be a useful method to isolate phenolic compounds from rapeseed meal, but further optimisation and validation are necessary.

PEF extraction

PEF extraction is a process that does not require heat but generates an electric field, which results in the electroporation of the cellular membrane and the release of the bioactive constituents from the "protoplasm". The efficacy of this technique depends on various factors, including the strength of the electric field as well as the duration and number of pulses. A recent study (Teh *et al.*, 2015) optimised the extraction of polyphenols using PEF from rapeseed cake. In order to recover the maximum yield of polyphenols, the optimal parameters for PEF extraction were found to be as follows: voltage of 30 V, frequency of 30 Hz, 10% ethanol and an exposure time of 10 s. This method also proved advantageous over traditional methods due to the shorter extraction time and low solvent use (Teh *et al.*, 2015). Yu *et al.* (2016) evaluated PEF-assisted extraction of polyphenols in order to valorise a by-product of rapeseed oil production, rapeseed stem or green biomass. They determined that the number of pulses that yielded higher total polyphenol content of juices pressed at 10 b was 200, with a strength of 8 kv/cm. The efficacy of PEF pre-treatment was demonstrated by the increase in total polyphenols with PEF pre-treatment and subsequent pressing. Without PEF pre-treatment, after 30 min of pressing, the result was 24% polyphenol yield from rapeseed. PEF pre-treatment with 200 pulses and an increase in strength to 8 kv/cm resulted in maximum polyphenol yield for pressing (Yu *et al.*, 2016). These initial studies have had success in isolating phenolics from rapeseed by-products, and it could be a promising novel method to isolate phenolic compounds; however, further studies are necessary to fully validate this method for use with rapeseed meal.

HVED approach

HVED is based on "electrical breakdown in water", which is accompanied by various phenomena including high-amplitude pressure shock waves and cavitation bubbles. This subsequently leads to the breakdown of cellular structures and the release of the cell contents. The use of HVED to isolate polyphenols from rapeseed cake was also investigated previously (Barba *et al.*, 2015). The study found that when isolating polyphenols from rapeseed cake, the liquid-to-solid ratio was a key parameter. The highest polyphenol content was observed using a liquid-to-solvent ratio of 20, while a liquid-to-solvent ratio of either 5 or 20, combined with HVED treatment at 80 kJ/kg also resulted in increased polyphenol content. However, it was noted that higher energy inputs >80 kJ/kg in rapeseed cake led to a decrease in polyphenol content. The authors suggest that high-energy inputs result in the creation of radicals, which in turn decrease the polyphenol content (Barba *et al.*, 2015). Crucially, it was found that the extracts from rapeseed cake had a significant increase in their antioxidant activity following HVED treatments. Other studies have shown the efficacy of this technique to extract phenolics from different oilseed sources, including sesame cake and flaxseed cake (Boussetta *et al.*, 2013; Sarkis *et al.*, 2015). The study by Barba *et al.* was an initial optimisation, in addition to being the first step in the search for more efficient methods to isolate phenolic compounds from rapeseed by-products. Further optimisation and validation are necessary in order to ensure the efficacy of this novel technique; however, it is worthy of mention due to its proven ability to extract phenolic compounds and its apparent efficiency over traditional methods, such as lower solvent consumption.

Characterisation strategies for rapeseed meal phenolics

As SA and PCA found in rapeseed possess various bioactive properties, including anti-inflammatory, anti-diabetes and anti-cancer activities, they are of interest as functional food components (Cherng *et al.*, 2013; Del Corno *et al.*, 2014; Tsao *et al.*, 2014). Therefore, further separation and purification are necessary to isolate these phenolic compounds from the initial fractions. Initially, the total phenolic content is often quantified using the Folin-Ciocalteu assay (Vuorela, 2005). Chemical characterisation and quantification are commonly performed using high-performance liquid chromatography (HPLC) or gas–liquid chromatography (GLC)/gas chromatography–mass spectrometry (GC-MS) (Cai and Arntfield, 2001; Canini *et al.*, 2007; Khattab *et al.*, 2010). HPLC is the preferred method for separation and quantification of phenolic compounds due to the fact that there is a wide range of commercially available columns, as well as the potential to combine two or more columns with differing column chemistries (Stalikas, 2007). However, despite the fact that HPLC is a commonly used technique, it has been suggested that GC-MS is more efficient, providing improved resolution and baseline separation with minimum co-elution. It must also be noted that when quantifying phenolic compounds with GC, there is sometimes a requirement for chemical modification, such as transformation to more volatile derivatives (Khoddami *et al.*, 2013).

SA and PCA in human health and disease

Properties of SA

Antioxidant activity of SA

SA can modulate oxidative stress pathways in the cell and may have potential in treating diabetes and high blood pressure (Roy and Prince, 2012; Silambarasan *et al.*, 2015). Oxidative stress can be defined as an imbalance in the "redox" status of a cell, which occurs when the levels of reactive oxygen species (ROS) and antioxidants are unbalanced (Nordberg and Arner, 2001). ROS can be found extrinsically in the form of pollutants, tobacco smoke and radiation; however, ROS may also be generated intrinsically through various pathways in the mitochondria (Trachootham *et al.*, 2009). The single electron reduction of molecular oxygen leads to the formation of a superoxide anion, a molecule that acts as a precursor for the formation of most other ROS (Turrens, 2003). In order to maintain homeostatic levels of reactive superoxide anions, the mitochondrial matrix contains a family of metalloenzymes known as superoxide dismutases (SODs). Compounds such as ascorbic acid, tocopherols and glutathione (GSH) also provide antioxidant activity (Jalaludeen and Pari, 2011). High levels of ROS are known to be carcinogenic, damaging vital cellular components including lipids, proteins and DNA. Given the destructive potential of ROS, it is generally thought that the use of antioxidants could reduce intracellular ROS levels and thus alleviate ROS-mediated damage (Brieger *et al.*, 2012). Recent studies indicate the antioxidant activity of SA and SA's superoxide radical and hydroxyl radical, as well as its $-O_2^{\cdot}$-scavenging activity (Zou *et al.*, 2002; Jalaludeen and Pari, 2011). Using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, it was found that SA had higher free radical-scavenging activity than its alkyl ester methyl sinapate or the control Trolox, a vitamin E analogue. Ferric reducing antioxidant power (FRAP) assays also revealed the higher antioxidant scavenging activity of SA compared to its alkyl ester methyl sinapate or the control Trolox (Gaspar *et al.*, 2010). The mechanism and kinetics of SA's antioxidant activity were measured using its hydroperoxyl radical (HOO•)-scavenging activity in lipidic and aqueous solutions, which occurs through the transfer of a hydrogen atom from its -OH group. The anionic form of SA at physiological pH was also more reactive than its neutral form towards oxygenated radicals (Galano *et al.*, 2011).

Histone deacetylase inhibition

Histone deacetylases (HDACs) remove acetyl groups from histones, resulting in a "closed" chromatin conformation that blocks gene transcription and therefore gene expression. Aberrant HDAC activity is common in various cancer types, driving carcinogenesis through the repression of genes involved in the cell cycle regulation, cellular differentiation and apoptosis (Tortorella *et al.*, 2015). Thus, HDAC inhibitors are emerging as potential therapeutic agents. There has been success in developing clinical therapeutics using HDAC inhibitors from both natural and synthetic sources. For example, suberoylanilide hydroxamic acid (SAHA) (vorinostat) and the natural product, romidepsin, were both approved for the treatment of cutaneous T-cell lymphoma (Mair *et al.*, 2014). SA was previously shown to have HDAC inhibitory activity. Senawong *et al.* (2013) extracted phenolic compounds from the rhizome of *Hydnophytum formicarum* Jack, and SA was found to be one of the major components. SA was found to block HDAC enzyme activity *in vitro* at a similar concentration as the established HDAC inhibitor sodium butyrate, with the authors suggesting that SA partly accounted for the HDAC inhibitory activity of the phenolic extract. The phenolic extract possessing HDAC inhibitory activity was found to inhibit the growth of HeLa cells in a dose- and time-dependent manner, while SA was also found to significantly inhibit the growth of HT-29 cells. The phenolic extract and SA were also found to have a significant effect on the induction of apoptosis, up to 78.9% and 8.4%, respectively.

Anti-cancer and antibacterial activities

SA was found to be cytotoxic against various cancer cell lines, including the human colon cancer cell lines HT-29 and SW480 and the human laryngeal carcinoma Hep-2 cells, when assayed previously (Balaji *et al.*, 2014; Janakiraman *et al.*, 2014). The cytotoxicity is probably due to the anti-proliferative activity of SA, which is supported by a study demonstrating SA's anti-proliferative activity against the breast cancer cell line T47D (Kampa *et al.*, 2004). The antibacterial effect of SA was demonstrated to be anti-carcinogenic, brought about by decreasing mucinase activity, an enzyme secreted by gut microflora. This inhibited mucin degradation and enhanced contact between carcinogens and colon epithelial cells. SA also inhibited carcinogenesis by increasing the activities of enzymatic and non-enzymatic antioxidants, including SOD, catalase (CAT) and GSH, with ROS known to play a role in early carcinogenesis. SA could potentially prevent the onset of carcinogenesis, as demonstrated in a study by Balaji *et al.* (2015). Colon cancer is the third most common malignancy worldwide, with a high-fat diet being a primary risk factor for developing colon cancer. Consumption of a high-fat diet has been reported to increase the development of pre-neoplastic lesions. Aberrant crypt foci (ACFs) are pre-neoplastic lesions, which are formed during an early event in carcinogenesis in humans and rodents. Rats fed a high-fat diet consisting of peanut oil had increased crypt numbers and "multiplicity". SA supplementation in 1,2-dimethylhydrazine (DMH)-induced colon cancer rats decreased the development and number of ACFs. SA also decreased β-glucuronidase activity in DMH-induced rats. Increased β-glucuronidase activity is

considered a primary causative factor in colon cancer, via the formation of methylazoxymethanol, a metabolite that induces the formation of DNA adducts. SA was effective in reducing the development of pre-cancerous lesions when studied previously (Balaji *et al.*, 2015).

Protective role in cardiovascular disease

Cardiovascular disease is the number one cause of death globally and the leading cause of death among Europeans (Townsend *et al.*, 2015). The available figures from 2013 revealed that 30.8% of deaths in the US were due to cardiovascular disease (Mozaffarian *et al.*, 2016). Lifestyle modifications, including a healthy diet, can play a protective role. Although no human studies have been performed, SA was shown to have a protective effect on isoproterenol (ISO)-induced myocardial infarction (MI) in rats. MI induction with ISO is a well-established animal model used to evaluate the effects of various cardioprotective agents because the pathophysiological changes are analogous to those taking place in humans with MI. Pre- or co-treatment with SA normalised the activity of serum creatine kinase (CK-MB), suggesting that SA protects the heart and reduces the damage leading to leakage of CK-MB. SA normalised the levels of lipid peroxidation products, such as cardiac malondialdehyde, and as such, apparently inhibited oxidative stress and stabilised lysosomal membranes. In accordance with this finding, SA normalised lysosomal enzymes including β-glucuronidase and β-galactosidase, as well as cathepsins B and D. Co-treatment with SA also reduced the size of the infarct, protecting the heart from ISO-induced MI (Roy and Prince, 2012).

Reperfusion injury occurs due to ischaemia followed by reintroduction of blood flow to a previously ischaemic tissue, causing additional myocardial damage and cardiac contractile dysfunction (Silambarasan *et al.*, 2015). As oxidative stress is an important contributor to ischaemia/reperfusion (I/R) injury, antioxidants have been considered as a potential therapeutic option (Yang *et al.*, 2013). Oral pre-treatment with SA significantly improved the percent rate–pressure product and percent coronary flow when compared to untreated I/R hearts. Mitochondrial dysfunction results in cardiac contractile dysfunction and hypertrophy; moreover, under conditions of oxidative stress, the enzymes of the tricarboxylic acid (TCA) cycle are disrupted. Decreases in the activity of several TCA enzymes were blunted after pre-treatment with SA. SA was also found to reduce H9c2 cardiomyoblast cell injury caused by H_2O_2, probably due to its scavenging abilities (Silambarasan *et al.*, 2015).

In N^{ω}–nitro-l-arginine methyl ester hydrochloride (L-NAME)-treated rats, SA decreased the mean arterial pressure, along with kidney and liver weights. SA increased the activities of the antioxidant enzymes SOD and CAT, along with the levels of non-enzymatic antioxidants such as the vitamins C and E. Ventricular hypertrophy was observed due to L-NAME treatment, causing a reduction in contractile function. SA treatment restored this ventricular function, which the authors attributed to its antioxidant abilities, considering that previous studies have shown that oxidative stress plays a role in fibrosis during hypertension. Damaged cells leak hepatic enzymes such as aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT), causing an increase in their activities. Treatment with SA decreased their activities and controlled liver damage, which was validated by histopathological examination of the liver. SA was also found to decrease renal function markers, including urea, uric acid and creatinine, towards normal levels. Epidemiological studies have linked hypercholesterolaemia to hypertension. Furthermore, SA treatment was found to lower total cholesterol levels in the liver and kidney of L-NAME spontaneously hypertensive rats. Docking studies revealed that this effect was due to the repression of endogenous cholesterol biosynthesis by inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (Silambarasan *et al.*, 2016).

Anti-diabetes activity

Diabetes is a group of "heterogeneous, hormonal and metabolic disorders", with hyperglycaemia being one of its physiological characteristics. Type 2 diabetes (T2D), in particular, is one of the most prevalent "metabolic pathologies" worldwide, with >220 million people affected. Chronic hyperglycaemia has detrimental cellular effects, as oxidation of glucose as well as protein glycation and glycoxidation all enhance the formation of ROS. The biochemical and physiological effects of SA treatment on streptozotocin (STZ)-induced diabetic rats revealed that oral administration of SA was found to cause a significant improvement in body weight, attributed to the anti-hyperglycaemic effect of SA (Stumvoll *et al.*, 2005). SA also influenced other physiological parameters, such as decreasing kidney weight to near-normal values. SA significantly decreased plasma glucose when compared to diabetic control rats, which the authors suggest could be due to the regeneration of existing pancreatic β-cells by SA and enhanced transport of glucose to peripheral tissues. SA significantly increased the levels of both C-peptide and insulin in STZ-induced diabetic rats, while decreasing the level of glycosylated haemoglobin (HbA1c) and increasing the level of total haemoglobin. The authors speculate that these changes could be due to the reduction of blood glucose level. Increased activities of enzymes involved in gluconeogenesis, i.e. glucose-6-phosphatase and fructose-1, 6-biphosphatase, were observed in the liver and kidney of diabetic rats. SA restored their level of activity to near normal, resulting in improved glycaemic control (Kanchana *et al.*, 2011).

SA can directly influence glucose utilisation, by significantly lowering postprandial glucose without altering plasma insulin levels. Glucose transporter type 4 (GLUT4) expression was significantly increased in the soleus muscle of STZ-induced rats, with SA causing a concentration-dependent increase in glucose uptake in the soleus muscle. This was confirmed in L6 myoblast cells, indicating that SA may directly stimulate glucose uptake. *In vivo*, SA increased insulin sensitivity, probably due to the enhanced glucose uptake in skeletal muscle (Cherng *et al*., 2013). Taken together, these results indicate that SA may be clinically useful in the treatment of diabetes.

Anti-inflammatory activity

Nuclear transcription factor kappa-B (NF-κB) plays a key role in the pathogenesis of inflammation, and as such, there are numerous drugs designed to treat inflammatory disease, which are based on the inhibition of NF-κB, e.g. non-steroidal anti-inflammatory drugs. In response to lipopolysaccharide (LPS), SA inhibited the expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, which were markedly upregulated (Yun *et al.*, 2008). The mRNA levels of both iNOS and COX-2 were also reduced, indicating transcriptional inhibition. SA reduced the production of tumour necrosis factor (TNF)-α and interleukin (IL)-1β, as well as their mRNA expression levels. RAW 264.7 cells treated with SA had a reduction in the DNA-binding activity of p65, preventing transcription initiation by NF-κB and subsequent expression of genes involved in the immune response (Schmitz and Baeuerle, 1991; Pahl, 1999). The nuclear translocation of both p65 and p50 was also inhibited. Another key finding was SA's ability to inhibit the LPS-induced degradation of I kappa B-alpha (IkB-α), preventing the release of NF-κB. These results demonstrate that SA inhibits iNOS, TNF-α, COX-2 and IL-1β expression, and this is at least partly mediated via an NF-κB mechanism. This anti-inflammatory activity was also demonstrated *in vivo*, with SA inhibiting oedema caused by acute inflammation in serotonin- and carrageenan-induced rat paw oedema models (Yun *et al.*, 2008).

Properties of PCA

Antioxidant activity of PCA

The physiological effect of PCA is believed to be linked to its antioxidant activities, which were recently investigated in vitro. DPPH and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assays demonstrated that PCA had better scavenging activity than the positive controls Trolox and butylated hydroxytoluene (BHT). When compared to Trolox and BHT in terms of reducing power percentage (%-Fe^{3+} and %-Cu^{2+}), PCA was found to be much more efficient. PCA also had lower half-maximal inhibitory concentration (IC_{50}) values for both Fe^{3+} and Cu^{2+} assays, in comparison to Trolox and BHT. It was found that PCA generally exhibited higher inhibition levels than Trolox, but not BHT, in terms of scavenging of superoxide anion and hydroxyl radical. The generally lower IC_{50} values obtained for PCA in comparison to the positive controls Trolox and BHT indicate that PCA is a better antioxidant, as a lower IC_{50} value indicates higher antioxidant activity. The DPPH and ABTS assays were conducted in organic solutions, while the reducing power (Fe^{3+} and Cu^{2+}) and superoxide anion- and hydroxyl radical-scavenging assays were carried out in aqueous solutions. Therefore, PCA exhibited significant antioxidant abilities in both lipidic and aqueous media and is thus considered an ideal natural antioxidant in both media (Li *et al.*, 2011). The potent antioxidant activity of PCA was also shown in ageing rats. Treatment with PCA caused a significant rise in the enzymatic activities of CAT and GSH peroxidase (GSH-PX), both of which are endogenous enzymatic antioxidants (Zhang *et al.*, 2011). This highlights the therapeutic potential for PCA in the treatment of age-associated disorders, such as cancer, through decreasing the levels of harmful ROS.

HDAC inhibition

Khaopha *et al.* (2015) obtained phenolic-rich testa extracts from peanut testae (skins), which were found to possess HDAC inhibitory activity and anti-cancer activity. PCA and *p*-coumaric acid were found to be the most predominant phenolic acids, with the highest quantity of PCA in the ICG15042 extract, while SA was greater in the KK4 extract (Khaopha *et al.*, 2015). HeLa cells treated with the phenolic-rich testa extracts ICG15042, KS2 and KK4 displayed a significant increase in all four acetylated lysine residues of histone H4, indicating HDAC inhibition. The authors suggested that some of the phenolic compounds in the extracts were functioning as HDAC inhibitors, with extracts ICG15402 and KK4 chosen for further evaluation as they displayed the highest HDAC inhibitory activity. Both extracts significantly inhibited the proliferation of various human cancer cell lines, including HeLa (human cervical adenocarcinoma), HT29 (human colon adenocarcinoma), HCT116 (human colorectal carcinoma) and Jurkat (human T-cell leukaemia) cells, with Jurkat cells being particularly sensitive to this anti-proliferative activity. Both extracts induced apoptosis in the cancer cell lines; however, it appeared that apoptotic induction was most effective in the HeLa and HCT116 cells, a result also observed by Senawong *et al.* (2013). Importantly, when the extracts were tested with a non-cancer cell line, the number of apoptotic cells was similar to that with the solvent control, indicating that the phenolic extracts were not harmful to normal cells (Khaopha *et al.*, 2015). This is crucial given that specificity is a vital attribute to any therapeutic agent, in order to minimise damage to healthy cells and reduce side effects.

Anti-cancer activity

Cytotoxicity

PCA is cytotoxic to various cancer cell lines, including human breast MCF-7 cells, lung cancer A549 cell, HepG2 hepatocellular carcinoma, cervix HeLa and prostate cancer LNCaP cells. PCA was able to penetrate the cancer cells, destroying the integrity of the plasma membrane, causing apoptosis and lactate dehydrogenase (LDH) leakage. PCA also decreased Na+/K+-ATPase activity, thereby disrupting ion homoeostasis, resulting in collapse of the mitochondrial membrane. This collapse resulted in the release of pro-apoptotic factors, initiating the intrinsic apoptotic pathway, and was also suggested to cause an increase in the activities of caspase-8 and -3, which are key mediators of apoptosis (Yin *et al.*, 2009). PCA also displayed cytotoxicity against non-small cell lung cancer via similar mechanisms as reported herein. They found that PCA induced mitochondrial dysfunction and apoptosis through increases in Bax and caspase-3 expression and a decrease in Na+/K+-ATPase activity. PCA also modulated several signalling pathways, including FAK, MAPK and NF-κB pathways and thus reduced the release of inflammatory cytokines such as IL- 6 and IL-8. Production of proteins involved in metastasis (such as basic fibroblast growth factor [bFGF] and the matrix metalloproteinases MMP-2 and MMP-9) were also reduced (Tsao *et al.*, 2014).

Chemoprevention and reduced side effects

Cancer chemoprevention can be defined as the prevention of cancer using non-toxic synthetic chemicals or chemicals from natural substances before malignancy occurs. PCA was previously shown to prevent oral carcinogenesis, reducing incidences of neoplasms in the tongue (squamous cell papilloma and carcinoma), along with significantly decreasing pre-neoplastic lesions (hyperplasia and dysplasia) (Tanaka *et al.*, 1994). Anthocyanins derived from black raspberries, along with the main anthocyanin metabolite PCA, were studied in *N*-nitrosomethylbenzylamine (NMBA)-induced oesophageal tumours in rats. PCA delayed the formation of pre-neoplastic lesions and the progression of these lesions to papillomas, ultimately reducing NMBA-induced oesophageal carcinogenesis. PCA also induced the expression of PTX3, which was shown to possess anti-angiogenic and anti-tumourigenic activity in prostate cancer cells (Peiffer *et al.*, 2014).

Although cancer prevention is crucial, improving existing therapies and reducing harmful side effects is just as vital. Cisplatin is widely used to treat various solid malignancies; however, its use is limited due to its associated nephrotoxicity, which primarily occurs in kidney proximal tubule epithelial cells (Karasawa and Steyger, 2015). PCA was found to prevent induced oxidative damage in kidney proximal tubule LLC-PK1 cells *in vitro*, while *in vivo* studies showed that PCA prevented cisplatin-induced increase in the renal function marker, creatine. Cisplatin treatment also resulted in distinct histopathological changes in the rat kidney, such as tubular epithelial cellular necrosis, desquamation, vacuolisation and swelling. PCA treatment caused notable improvement in the histological appearance, with reduced tubular cell damage and swelling, providing almost "complete renoprotection" (Yamabe *et al.*, 2015).

Anti-metastatic activity

Metastasis accounts for almost 90% of cancer-associated mortality and is the biggest problem in the clinical treatment and management of cancer. PCA represents a potentially important agent for those patients who are diagnosed with early cancer lesions, as the anti-metastatic effects of PCA could stop the subsequent dissemination from the primary tumour and prevent metastasis in those patients (Chaffer and Weinberg, 2011). The metastasis process is still poorly understood, and there is an urgent need to develop new therapeutic strategies and targets to prevent or inhibit this phenomenon. The anti-metastatic potential of PCA in the highly metastatic melanoma cell line B16/F10 and in human gastric carcinoma AGS cells was also demonstrated previously. PCA inhibited the secretion of MMP-2 in human gastric carcinoma AGS cells and inhibited the protein kinase B (Akt)/NF-κB/MMP-2 pathway via the activation of RhoB. Metastasis of the B16/F10 melanoma cells to the liver of C57/BL6 mice was also considerably reduced by PCA treatment (Lin *et al.*, 2011).

Anti-inflammatory activity

Studies have demonstrated the ability of PCA to inhibit the LPS-induced secretion of pro-inflammatory cytokines, such as IL-8, IL-2 and chemokine (C-C motif) ligand-2 (CCL2), from monocyte-derived dendritic cells and human gingival fibroblasts via activation of peroxisome proliferator-activated receptor (PPAR)-g. As cytokines such as IL-8 and CCL2 are involved in recruiting inflammatory cells, a reduction in the IL-8 content in the extracellular environment may limit the recruitment of inflammatory cells and thus prevent the initiation of an inflammatory immune response (Del Como *et al.*, 2014; Wang *et al.*, 2015b). PCA can also prevent inflammation by inhibiting the oxidation of fatty acids, which are activators of PPAR-g signalling (Nagy *et al.*, 1998). The pathology of neurodegenerative diseases, including Parkinson's and Alzheimer's disease (AD), has been attributed to the overproduction of inflammatory mediators such as TNF-α, IL-6 and IL-1β. These inflammatory mediators are produced following the activation of microglia, which are immune cells resident in the brain, by LPS, and the subsequent activation of NF-κB and MAPKs. As such, regulating the activity of

the microglia could be a potential therapeutic strategy. In accordance with this observation, Wang *et al.* (2015) found that PCA could inhibit the inflammatory mediators TNF-α, IL-1β, IL-6 and prostaglandin-2 (PGE2) but could also inhibit the LPS-induced activation of NF-κB and MAPKs, both of which are key regulators of inflammatory cytokine production. Another key finding was PCA's ability to inhibit the expression of Toll-like receptor 4 (TLR4), a receptor that activates NF-κB and MAPKs upon stimulation by LPS. This, therefore, suggests that PCA exerts its anti-inflammatory effects through inhibition of the TLR4 pathway, making it a potential candidate to regulate the activation of microglia in order to treat neurodegenerative diseases (Wang *et al.*, 2015a).

A recently published study has identified PCA as a potential therapeutic agent in the treatment of inflammatory bone disease. Osteoclasts are necessary for bone matrix degradation and to initiate bone re-modelling. However, an imbalance in their activity can lead to bone deterioration, which is a symptom of diseases such as osteoporosis and rheumatoid arthritis. M-CSF and RANK-L are potent inducers of mature osteoclasts, with RANK-L activating transcription factors such as c-Fos, which activate osteoclasts and induce their differentiation. PCA was found to inhibit RANK-L-induced c-Fos protein expression, while also inhibiting the differentiation of osteoclasts in mouse bone marrow macrophages. The bone-resorbing ability of mature osteoclasts was also inhibited. *In vivo*, PCA was shown to have a protective effect against LPS-induced bone loss. PCA was also found to downregulate the expression of osteoclastogenesis-related genes, including DC-STAMP and b3-integrin (Park *et al.*, 2016b).

Protective role in cardiovascular disease

The co-morbidities associated with diabetes pose serious health risks, with vascular complications being the primary cause of mortality and morbidity in those suffering from chronic diabetes. The effects of PCA on vascular responses in chronically diabetic rats were also examined. PCA was found to protect blood vessels by increasing nitric oxide production, which prevents the interaction of platelets and leucocytes with the vascular cell wall. Hypertension is a common complication of diabetes. PCA was found to cause significant reductions in the high diastolic, systolic and mean arterial blood pressures observed in chronically diabetic rats. PCA also ameliorated diabetes-induced high blood pressure by restoring the reduced vascular reactivity in the diabetic rats (Semaming *et al.*, 2016). This improved reactivity was exemplified by the restoration of the vasoconstriction and vasodilation responses following PCA treatment. PCA treatment also caused a decrease in the oxidative stress marker malondialdehyde (MDA), while the activities of the antioxidant enzymes SOD and CAT were increased. The authors, therefore, suggested that the improved vascular reactivity observed in the diabetic rats could be due to the antioxidant activity of PCA, given that it has been postulated that oxidation plays a role in vascular impairment in diabetes. The potential of PCA to prevent or limit cardiac complications in diabetes was demonstrated earlier using type 1 diabetic rats by Semaming *et al.* (2014), who found that PCA reduced MDA levels, along with attenuating mitochondrial depolarisation, decreasing mitochondrial swelling and attenuating mitochondrial ROS production. PCA in combination with insulin could restore echocardiography to a normal status, which could not be achieved by insulin alone. HbA1c levels were also reduced, indicating greater long-term glycaemic control. Cardiac apoptosis is a poor predictor of outcome in those with cardiac disease or heart failure. In line with this, PCA increased the anti-apoptotic protein Bcl2 in the heart tissue of diabetic rats (Semaming *et al.*, 2014).

Currently, there is a need for anticoagulation agents from natural sources in the clinic. In addition, the anti-thrombotic potential of PCA derived from fermented pine needles was evaluated. The processes of coagulation and anticoagulation are tightly regulated, and an imbalance in this process can lead to thrombus formation, which adheres to blood vessel walls. If this thrombus is not broken down, circulation is interrupted, leading to high blood pressure, stroke, ischaemic heart disease and myocardial infarction. PCA was tested for its fibrinolysis activity, by measuring the turbidity in a fibrin clot solution. PCA caused a dramatic decrease in turbidity, indicating that the fibrin clot had dissolved. Further studies aimed to elucidate the mechanism behind the fibrinolytic activity of PCA. A pH range of 2–4 was optimal for PCA to exert its fibrinolytic activity. At this acidic pH, PCA mainly exists in a non-ionised form, therefore allowing it to form greater number of hydrophobic interactions. The authors suggested that this increased tendency towards hydrophobic interactions allows enhanced binding between PCA and polypeptide chains, compromising their stability and causing "loosening of the fibrin network". The authors concluded that PCA exerted its fibrinolytic activity through a "dissolving effect", rather than by degradation of fibrin and fibrinogen. *In vivo*, PCA was shown to significantly inhibit thrombosis induced by carrageenan in mouse tail when compared to the control. In $FeCl_3$-induced carotid arterial thrombus model experiments, rats treated with PCA had no thrombus formation, indicating that PCA could inhibit vascular obstruction (Park *et al.*, 2016a).

Anti-diabetes activity

Functional foods provide a promising alternative therapeutic strategy to drive positive metabolic changes that would impair the T2D process; however, few studies have elucidated the molecular mechanisms of polyphenols on insulin signalling. A recent study (Scazzocchio *et al.*, 2011) has shown that PCA is able to partially counteract the loss of insulin sensitivity in T2DM and activate the insulin receptor substrate-1/

phosphatidylinositol 3-kinase/Akt (IRS-1/PI3K/Akt) signalling pathway, leading to increased glucose uptake. In adipocytes, PCA was found to be dependent on activation of the entire insulin pathway, as inhibition of the major players in the insulin signalling cascade IRS-1, PI3K or Akt prevented glucose uptake and GLUT4 translocation. PCA has also been reported to enhance glucose uptake by increasing translocation of GLUT4 to the cell membrane or, additionally, by increasing adiponectin secretion, a protein hormone capable of modulating glucose and fatty acid metabolism (Scazzocchio *et al.*, 2011).

The effects of PCA on adenosine monophosphate (AMP)-activated protein kinase (AMPK) activation and the role of PCA in glucose homoeostasis in mice were examined previously (Talagavadi *et al.*, 2016). AMPK is involved in metabolic regulation and was reported to be central to the control of glucose metabolism. In contrast, mammalian target of rapamycin (mTOR) and S6K1 are known to play a role in insulin resistance. It was found that PCA could significantly activate AMPK, although indirectly, and downregulate S6K in mouse livers. Upon intraperitoneal administration of glucose, there was a significant decrease in the glucose level in PCA-treated mice, with similar results obtained for the positive control metformin, indicating that PCA can induce glucose tolerance. In obese mice, pre-treatment with PCA significantly reduced hyperglycaemia after glucose injection. Furthermore, the insulin tolerance test showed that PCA administration amplified the glucose decrease, indicating that PCA improved insulin sensitivity in obese mice (Talagavadi *et al.*, 2016).

Protective neurological effects

In order to repair injured nerves in adults, proliferation of nerve cells is a vital process. Schwann cells are crucial for the function and regeneration of neurons, and it was recently demonstrated that PCA could stimulate Schwann cell proliferation and survival by phosphorylating the insulin-like growth factor-I (IGF-I)-mediated PI3K/Akt pathway (Ju *et al.*, 2015). PCA enhanced the proliferation of RSC96 cells, inducing cell cycle progression at the G1-to-S phase. An increase in cyclin A was observed, resulting in the promotion of DNA replication and growth of RSC96 cells. A phenomenon that is vital to the repair and regeneration process of nerves is the migration of RSC96 Schwann cells along the growth direction. It was found that PCA enhanced Schwann cell migration, and that the IGF-1/Akt/PI3K pathway may play a key role in this process. The results of that study highlight the potential utility of PCA in the treatment of peripheral nerve injuries caused by trauma, surgery or acute compression. Guan *et al.* (2009) also demonstrated the ability of PCA to stimulate proliferation of rat hippocampal neural stem cells, which are believed to contribute to the regenerative potential of the adult central nervous system.

For patients suffering from neurodegenerative brain diseases, ameliorating symptoms is crucial. PCA was found to improve cognitive defects in a mouse model of AD and attenuate Ab deposition, which is a hallmark of AD (Song *et al.*, 2014). Inflammatory events in AD result from an imbalance in the formation and clearance of Ab, with mounting evidence that Ab accumulation is linked to the secretion of inflammatory cytokines. PCA reduced the levels of TNF-α, IL-1β, IL-6 and IL-8 in AbPP/PS1 mice. Although the underlying mechanism behind PCA's effect on Ab-induced neurotoxicity was not elucidated, it is attributed to its antioxidant and anti-inflammatory activities. Crucially, PCA displayed similar anti-inflammatory activity as donepezil, a cholinesterase inhibitor used to treat AD.

Protection against nephrotoxicity and hepatotoxicity

Cadmium (Cd) is a heavy metal that is a by-product of zinc production. It is considered to be one of the most toxic environmental and industrial pollutants. It can be ingested from foods, drinking water and contaminated soil or through cigarette smoking. The kidney is the most affected by prolonged low-level exposure to Cd, causing proteinuria, calciuria and tubular necrosis, all of which may result in end-stage renal failure, diabetic complications and osteoporosis. Cd can also lead to nephrotoxicity due to its tendency to settle in the proximal tubule of the nephron, although the underlying mechanism is yet to be fully elucidated. PCA can attenuate the nephrotoxicity and hepatotoxicity induced by Cd in rats. Treatment with Cd led to "severe" hepatic damage, evidenced by the increased activity of the hepatic enzymes alanine transaminase (ALT), AST and ALP in the serum of the Cd-induced hepatotoxicity group compared to the normal control. Co-treatment with PCA was found to ameliorate the activities of these hepatic enzymes. Crucially, PCA at doses of 10 and 20 mg/kg had no adverse effects on the rats (Adefegha *et al.*, 2015).

Potential use of SA and PCA recovered from rapeseed meal

SA and PCA may improve human health, and both compounds are present in traditional Chinese medicines, such as "Yinhuang powder" and *Salvia miltiorrhiza* (also known as Danshen) (Wen *et al.*, 2005; Cao *et al.*, 2014; Su *et al.*, 2015). Research on these phenolic compounds has suggested that they possess diverse bioactive properties, including anti-cancer, anti-diabetes and anti-inflammatory activities, in addition to a role in the prevention and treatment of cardiovascular and neurodegenerative diseases (Yun *et al.*, 2008; Balaji *et al.*, 2015; Ju *et al.*, 2015; Semaming *et al.*, 2016; Talagavadi *et al.*, 2016). Given these potent

bioactivities and the fact that rapeseed is a rich source of phenolic compounds, optimising methods to isolate these compounds from rapeseed meal is pertinent. SA and PCA in dietary sources are present in insufficient quantities to exert a sufficient physiological effect (Nunez-Sanchez *et al.*, 2015). Therefore, optimal extraction of these phenolics from rapeseed meal would allow them to be used as functional food ingredients and exert their physiological effects through dietary intake. The antioxidant properties of these phenolics would make them useful in the cosmetic industry, and indeed, a patent was filed regarding the use of SA as a cosmetic ingredient, such as in a topical agent to protect against the signs of skin ageing (Markert *et al.*, 2011).

Bottlenecks concerning the use of SA and PCA recovered from rapeseed meal

The stability and bioavailability of rapeseed phenolics is an issue in terms of the biorefining process and total utilisation of the rapeseed crop (Nićiforović and Abramovič, 2014; Dias *et al.*, 2015). Phenolic compounds are susceptible to spontaneous oxidation and degradation (Tan *et al.*, 2011). Oxidation may occur through enzymatic or non-enzymatic reactions, resulting in oxidation products that are "nutritionally unavailable" (Shahidi and Naczk, 1992). Phenolics can also interact with minerals, such as iron, and inhibit their absorption, and the metabolism of phenolics by phase II enzymes also leads to poor bioavailability (Vuorela, 2005; De Souza *et al.*, 2015). Bioavailability is a key issue, given that little is known about the metabolism of SA and PCA. The epithelium of the small intestine plays a role in the bioavailability and metabolism of SA, involving both phase I and phase II metabolic enzymes; however, compared to other hydroxycinnamic acids such as ferulic acid, little is known about the metabolism of SA (Nićiforović and Abramovič, 2014). A recent bioavailability study with rats fed the herb *Polygonum capitatum* found that PCA was primarily distributed in the kidney tissue, followed by the lung tissue; however, >80% was excreted (Ma *et al.*, 2016). PCA was not detected in the brain, indicating its inability to cross the blood–brain barrier; this is in contrast with a study by Guan *et al.* (2009), who detected PCA in rat brain micro-dialysates, indicating that it could cross the blood–brain barrier.

As such, validation of this rapeseed by-product requires stabilisation of the phenolic compounds before application in either food or cosmetics. Indeed, a patent regarding use of SA for cosmetics, including as a topical composition to reduce the signs of ageing, noted the instability of SA and proposed micro-encapsulation as a solution (Markert *et al.*, 2011). Nanoparticle- and microparticle-based delivery systems provide a potential solution to the problem of reduced stability and bioavailability of phenolics, including SA and PCA. Such systems were found to increase the phytochemical absorption of phenolic compounds in epithelial cells (Dias *et al.*, 2015). The increased bioavailability of resveratrol following micro-encapsulation is proof of the efficacy of this technique (Davidov-Pardo and McClements, 2014). Utilising SA and PCA as functional food ingredients allows validation of a rapeseed by-product and its potent bioactive phenolics. Phenolic compounds have previously been encapsulated using methods such as spray-drying, extrusion and atomisation/coagulation. Spray-drying is a very straightforward and common micro-encapsulation method used in the food industry. Carbohydrate polymers such as starch and cellulose are commonly used as the shell material and are good candidates as they are generally recognised as safe (GRAS) (Dias *et al.*, 2015). A recent *in vitro* study demonstrated the great potential to successfully encapsulate PCA for functional food purposes. Madureira *et al.* (2016) investigated chitosan nanoparticles "loaded" with PCA for potential incorporation into food matrices or as functional food ingredients. The study found that the best nanoparticle for PCA was a low-molecular-weight nanoparticle, and the encapsulation process did not diminish the antioxidant activity of PCA. It was found that the nanoparticles could bypass the acidic environment of the stomach intact and reach the intestine, where the neutral pH results in "coagulation/flocculation" of the chitosan nanoparticle and release of the phenolics. From the intestine, the phenolics can be absorbed into the bloodstream (Madureira *et al.*, 2016). Ultimately, validating this rapeseed by-product and its bioactive compounds for use in food, cosmetic and pharmaceutical industries depends on successful clinical trials. Currently, there are no clinical studies examining the bioavailability or bioactivity of phenolic acids from rapeseed, including SA and PCA. However, a search of Clinicaltrials.gov revealed that there have been trials on phenolic acids from other sources such as black tea, oat and purple wheat products in terms of their pharmacokinetics and bioavailability.

Conclusion

Phenolic compounds such as SA and PCA hold great promise due to their purported bioactive properties, including anti-diabetes, anti-cancer and anti-inflammatory activities, in addition to possessing a protective role in AD and cardiovascular disease. Various traditional methods are used to extract these phenolics from rapeseed meal, based on both solvents and enzymes. However, there are issues associated with these methods, including the requirement for large volumes of solvents and their associated toxicities. The need for simpler, more efficient methodologies has led to the development of novel techniques, such as ultrasound-

assisted, microwave-assisted, HVED and PEF extractions. Development of these novel techniques allows the validation of a rapeseed by-product and total utilisation of the rapeseed crop without generating waste. It must be noted that further optimisation and validation of these techniques are necessary with regard to isolating phenolic compounds from rapeseed meal.

In order to utilise the phenolic compounds isolated from rapeseed meal, there are issues that need to be addressed. The two major issues are stability and bioavailability, which could hinder the use of the phenolics in food and cosmetic industries. A solution is the micro-encapsulation of the phenolics, and initial studies have shown the efficacy of this technique, which would make the phenolics suitable for food applications. Ultimately, maximising the use of rapeseed meal phenolics depends on proven clinical safety and efficacy. To date, there are no clinical trials regarding rapeseed phenolics, including SA and PCA; however, there are clinical trials regarding the use of phenolic compounds from other sources.

References

Adefegha, S.A., Omojokun, O.S. and Oboh, G. 2015. Modulatory effect of protocatechuic acid on cadmium induced nephrotoxicity and hepatoxicity in rats in vivo. *Springerplus* **4**: 619.

Balaji, C., Muthukumaran, J. and Nalini, N. 2015. Effect of sinapic acid on 1,2 dimethylhydrazine induced aberrant crypt foci, biotransforming bacterial enzymes and circulatory oxidative stress status in experimental rat colon carcinogenesis. *Bratisl Lek Listy* **116**: 560–566.

Balaji, C., Muthukumaran, J., Vinothkumar, R. and Nalini, N. 2014. Anticancer Effects of Sinapic Acid on Human Colon Cancer Cell Lines HT-29 and SW480. *International Journal of Pharmaceutical and Biological Archives* **5**: 176–183.

Barba, F.J., Boussetta, N. and Vorobiev, E. 2015. Emerging technologies for the recovery of isothiocyanates, protein and phenolic compounds from rapeseed and rapeseed press-cake: effect of high voltage electrical discharges. *Innovative Food Science & Emerging Technologies* **31**: 67–72.

Booth, E.J. 2004. Extraction and refining. In: "Rapeseed and Canola Oil. Production, Processing, Properties and Uses" (ed. F.D. Gunstone), Blackwell Publishing, Oxford, UK, pages 17–36.

Boussetta, N., Turk, M., De Taeye, C., Larondelle, Y., Lanoiselle, J. L. and Vorobiev, E. 2013. Effect of high voltage electrical discharges, heating and ethanol concentration on the extraction of total polyphenols and lignans from flaxseed cake. *Industrial Crops and Products* **49**: 690–696.

Brieger, K., Schiavone, S., Miller, F.J. and Krause, K.H. 2012. Reactive oxygen species: from health to disease. *Swiss Medical Weekly* **142**: w13659.

Cai, R. and Arntfield, S.D. 2001. A rapid high-performance liquid chromatographic method for the determination of sinapine and sinapic acid in canola seed and meal. *Journal of the American Oil Chemists' Society* **78**: 903–910.

Canini, A., Alesiani, D., D'Arcangelo, G. and Tagliatesta, P. 2007. Gas chromatography–mass spectrometry analysis of phenolic compounds from *Carica papaya* L. leaf. *Journal of Food Composition and Analysis* **20**: 584–590.

Cao, J., Wei, J., Tian, K., Su, H., Wan, J. and Li, P. 2014. Simultaneous determination of seven phenolic acids in three Salvia species by capillary zone electrophoresis with beta-cyclodextrin as modifier. *Journal of Separation Science* **37**: 3738–3744.

Carre, A.P. 2014. Rapeseed market, worldwide and in Europe. *Oilseeds and Fats Crops and Lipids* **21**: 1–12.

Chaffer, C.L. and Weinberg, R.A. 2011. A perspective on cancer cell metastasis. *Science* **331**: 1559–1564.

Chemat, F., Vian, M.A. and Cravotto, G. 2012. Green extraction of natural products: concept and principles. *International Journal Molecular of Sciences* **13**: 8615–8627.

Cherng, Y.G., Tsai, C.C., Chung, H.H., Lai, Y.W., Kuo, S.C. and Cheng, J.T. 2013. Antihyperglycemic action of sinapic acid in diabetic rats. *Journal of Agricultural and Food Chemistry* **61**: 12053–12059.

Cheynier, V., Comte, G., Davies, K.M., Lattanzio, V. and Martens, S. 2013. Plant phenolics: recent advances on their biosynthesis, genetics, and ecophysiology. *Plant Physiology and Biochemistry* **72**: 1–20.

Cvjetko., M., Lepojevic., Ž., Zekovic., Z., Vidovic., S., Svetlana and Milosevic 2009. Antioxidant properties of rapeseed. *Proceedings of the Faculty of Technology in Leskovac, Leskovac, Serbia,* pages 27–33.

Davidov-Pardo, G. and McClements, D.J. 2014. Resveratrol encapsulation: designing delivery systems to overcome solubility, stability and bioavailability issues. *Trends in Food Science & Technology* **38**: 88–103.

De Ferrars, R.M., Czank, C., Zhang, Q., Botting, N.P., Kroon, P.A., Cassidy, A. and Kay, C.D. 2014. The pharmacokinetics of anthocyanins and their metabolites in humans. *British Journal of Pharmacology* **171**: 3268–3282.

De Souza, J.E., Casanova, L.M., and Costa, S.S. 2015. Bioavailability of phenolic compounds: a major challenge for drug development? *Revista Fitos, Rio de Janeiro* **9**: 1–72.

Del Corno, M., Varano, B., Scazzocchio, B., Filesi, C., Masella, R. and Gessani, S. 2014. Protocatechuic acid inhibits human dendritic cell functional activation: role of PPARgamma up-modulation. *Immunobiology* **219**: 416–424.

Dias, M.I., Ferreira, I.C.F.R. and Barreiro, M.F. 2015. Microencapsulation of bioactives for food applications. *Food & Function* **6**: 1035–1052.

Dubie, J., Stancik, A., Morra, M. and Nindo, C. 2013. Antioxidant extraction from mustard (*Brassica juncea*) seed meal using high-intensity ultrasound. *Journal of Food Science* **78**: E542–E548.

Galano, A., Francisco-Marquez, M. and Alvarez-Idaboy, J.R. 2011.

Mechanism and kinetics studies on the antioxidant activity of sinapinic acid. *Physical Chemistry Chemical Physics* **13**: 11199–11205.

Gaspar, A., Martins, M., Silva, P., Garrido, E.M., Garrido, J., Firuzi, O., Miri, R., Saso, L. and Borges, F. 2010. Dietary phenolic acids and derivatives. Evaluation of the antioxidant activity of sinapic acid and its alkyl esters. *Journal of Agricultural and Food Chemistry* **58**: 11273–11280.

Guan, S., Ge, D., Liu, T.Q., Ma, X.H. and Cui, Z.F. 2009. Protocatechuic acid promotes cell proliferation and reduces basal apoptosis in cultured neural stem cells. *Toxicology In Vitro* **23**: 201–208.

Jalaludeen, A.M. and Pari, L. 2011. Studies on the antioxidant and free radical-scavenging effect of sinapic acid: an in vivo and in vitro model. *Journal of Pharmaceutical Sciences and Research* **3**: 1447–1455.

Janakiraman, K., Kathiresan, S. and Mariadoss, A.V. 2014. Influence of sinapic acid on induction of apoptosis in human laryngeal carcinoma cell line. *International Journal of Modern Research and Reviews* **2**: 165–170.

Ju, D.T., Liao, H.E., Shibu, M.A., Ho, T.J., Padma, V.V., Tsai, F.J., Chung, L.C., Day, C.H., Lin, C.C. and Huang, C.Y. 2015. Nerve regeneration potential of protocatechuic acid in RSC96 Schwann cells by induction of cellular proliferation and migration through IGF-IR-PI3K-Akt signaling. *Chinese Journal of Physiology* **58**: 412–419.

Kakkar, S. and Bais, S. 2014. A review on protocatechuic acid and its pharmacological potential. *ISRN Pharmacology* **2014**: 9.

Kampa, M., Alexaki, V.I., Notas, G., Nifli, A.P., Nistikaki, A., Hatzoglou, A., Bakogeorgou, E., Kouimtzoglou, E., Blekas, G., Boskou, D., Gravanis, A. and Castanas, E. 2004. Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. *Breast Cancer Research* **6**: R63–R74.

Kanchana, G., Shyni, W.J., Rajadurai, M. and Periasamy, R. 2011. Evaluation of antihyperglycemic effect of sinapic acid in normal and streptozotocin-induced diabetes in albino rats. *Global Journal of Pharmacology* **5**: 33–39.

Karasawa, T. and Steyger, P.S. 2015. An integrated view of cisplatin-induced nephrotoxicity and ototoxicity. *Toxicology Letters* **237**: 219–227.

Khaopha, S.J., Patanothai, A. and Senawong, T. 2015. Histone deacetylase inhibitory activity of peanut testa extracts against human cancer cell lines. *Journal of Food Biochemistry* **39**: 263–273.

Khattab, R., Eskin, M., Aliani, M. and Thiyam, U. 2010. Determination of sinapic acid derivatives in canola extracts using high-performance liquid chromatography. *Journal of the American Oil Chemist's Society* **87**: 147–155.

Khoddami, A., Wilkes, M.A. and Roberts, T.H. 2013. Techniques for analysis of plant phenolic compounds. *Molecules* **18**: 2328–2375.

Kozlowska, H., Naczk, M., Shahidi, F. and Zademowski, R. 1990. Phenolic acids and tannins in rapeseed and canola. In: "Canola and Rapeseed" (ed. F. Shahidi), Springer, New York, US, pages 193–210.

Li, X., Wang, X., Chen, D. and Chen, S. 2011. Antioxidant activity and mechanism of protocatechuic acid in vitro. *Functional Foods in Health and Disease* **7**: 232–244.

Lin, H.H., Chen, J.H., Chou, F.P. and Wang, C.J. 2011. Protocatechuic acid inhibits cancer cell metastasis involving the down-regulation of Ras/Akt/NF-kappaB pathway and MMP-2 production by targeting RhoB activation. *British Journal of Pharmacology* **162**: 237–254.

List, G. 2015. Rapeseed (canola) oil. *Lipid Technology* **27**: 120.

Liu, Q., Wu, L., Pu, H., Li, C. and Hu, Q. 2012. Profile and distribution of soluble and insoluble phenolics in Chinese rapeseed (*Brassica napus*). *Food Chemistry* **135**: 616–622.

Lomascolo, A., Uzan-Boukhris, E., Sigoillot, J.C. and Fine, F. 2012. Rapeseed and sunflower meal: a review on biotechnology status and challenges. *Applied Microbiology and Biotechnology* **95**: 1105–1114.

Ma, F.-W., Deng, Q.-F., Zhou, X., Gong, X.-J., Zhao, Y., Chen, H.-G. and Zhao, C. 2016. The tissue distribution and urinary excretion study of gallic acid and protocatechuic acid after oral administration of polygonum capitatum extract in rats. *Molecules* **21**: 399.

Madureira, A.R., Pereira, A. and Pintado, M. 2016. Chitosan nanoparticles loaded with 2,5-dihydroxybenzoic acid and protocatechuic acid: properties and digestion. *Journal of Food Engineering* **174**: 8–14.

Mair, B., Kubicek, S. and Nijman, S. M. B. 2014. Exploiting epigenetic vulnerabilities for cancer therapeutics. *Trends in Pharmacological Sciences* **35**: 136–145.

Masella, R., Santangelo, C., D'Archivio, M., Li Volti, G., Giovannini, C. and Galvano, F. 2012. Protocatechuic acid and human disease prevention: biological activities and molecular mechanisms. *Current Medicinal Chemistry* **19**: 2901–2917.

Mozaffarian, D., Benjamin, E.J., Go, A.S., Arnett, D.K., Blaha, M.J., Cushman, M., Das, S.R., De Ferranti, S., Despres, J.P., Fullerton, H.J., Howard, V.J., Huffman, M.D., Isasi, C.R., Jimenez, M. C., Judd, S.E., Kissela, B.M., Lichtman, J.H., Lisabeth, L.D., Liu, S., Mackey, R.H., Magid, D.J., Mcguire, D.K., Mohler, E.R., Moy, C.S., Muntner, P., Mussolino, M.E., Nasir, K., Neumar, R.W., Nichol, G., Palaniappan, L., Pandey, D.K., Reeves, M.J., Rodriguez, C.J., Rosamond, W., Sorlie, P.D., Stein, J., Towfighi, A., Turan, T.N., Virani, S.S., Woo, D., Yeh, R.W. and Turner, M.B. 2016. Executive summary: heart disease and stroke statistics-2016 update: a report from the American Heart Association. *Circulation* **133**: 447–454.

Naczk, M., Wanasundara, P. K. J. P. D. and Shahidi, F. 1992. Facile spectrophotometric quantification method of sinapic acid in hexane-extracted and methanol-ammonia-water-treated mustard and rapeseed meals. *Journal of Agricultural and Food Chemistry* **40**: 444–448.

Nagy, L., Tontonoz, P., Alvarez, J. G. A., Chen, H. and Evans, R.M. 1998. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARγ. *Cell* **93**: 229–240.

Nićiforović, N. and Abramovič, H. 2014. Sinapic acid and its derivatives: natural sources and bioactivity. *Comprehensive Reviews in Food Science and Food Safety* **13**: 34–51.

Nordberg, J. and Arner, E.S. 2001. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radical Biology and Medicine* **31**: 1287–1312.

Nunez-Sanchez, M.A., Gonzalez-Sarrias, A., Romo-Vaquero, M., Garcia-Villalba, R., Selma, M.V., Tomas-Barberan, F.A., Garcia-Conesa, M.T. and Espin, J.C. 2015. Dietary phenolics against colorectal cancer – from promising preclinical results to poor translation into clinical trials: pitfalls and future needs. *Molecular Nutrition and Food Research* **59**: 1274–1291.

Oniszczuk, A. and Olech, M. 2016. Optimization of ultrasound-assisted extraction and LC-ESI–MS/MS analysis of phenolic acids from *Brassica oleracea* L. var. sabellica. *Industrial Crops and Products* **83**: 359–363.

Pahl, H.L. 1999. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* **18**: 6853–6866.

Park, J., Lee, B., Choi, H., Kim, W., Kim, H.J. and Cheong, H. 2016a. Antithrombosis activity of protocatechuic and shikimic acids from functional plant *Pinus densiflora* Sieb. et Zucc needles. *Journal of Natural Medicine* **70**: 492–501.

Park, S.H., Kim, J.Y., Cheon, Y.H., Baek, J.M., Ahn, S.J., Yoon, K.H., Lee, M.S. and Oh, J. 2016b. Protocatechuic acid attenuates osteoclastogenesis by downregulating JNK/c-Fos/NFATc1 signaling and prevents inflammatory bone loss in mice. *Phytotherapy Research* **30**: 604–612.

Peiffer, D.S., Zimmerman, N.P., Wang, L.S., Ransom, B.W., Carmella, S.G., Kuo, C.T., Siddiqui, J., Chen, J.H., Oshima, K., Huang, Y.W., Hecht, S.S. and Stoner, G.D. 2014. Chemoprevention of esophageal cancer with black raspberries, their component anthocyanins, and a major anthocyanin metabolite, protocatechuic acid. *Cancer Prevention Research (Phila)* **7**: 574–584.

Robbins, R.J. 2003. Phenolic acids in foods: an overview of analytical methodology. *Journal of Agricultural and Food Chemistry* **51**: 2866–2887.

Roy, S.J. and Prince, S.P. 2012. Protective effects of sinapic acid on lysosomal dysfunction in isoproterenol induced myocardial infarcted rats. *Food and Chemical Toxicology* **50**: 3984–3989.

Sarkis, J.R., Boussetta, N., Blouet, C., Tessaro, I. C., Marczak, L. D. F. and Vorobiev, E. 2015. Effect of pulsed electric fields and high voltage electrical discharges on polyphenol and protein extraction from sesame cake. *Innovative Food Science & Emerging Technologies* **29**: 170–177.

Scazzocchio, B., Vari, R., Filesi, C., D'Archivio, M., Santangelo, C., Giovannini, C., Iacovelli, A., Silecchia, G., Li Volti, G., Galvano, F. and Maslla, R. 2011. Cyanidin-3-O-beta-glucoside and protocatechuic acid exert insulin-like effects by upregulating PPARgamma activity in human omental adipocytes. *Diabetes* **60**: 2234–2244.

Schmitz, M.L. and Baeuerle, P.A. 1991. The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. *Embo Journal* **10**: 3805–3817.

Semaming, Y., Kukongviriyapan, U., Kongyingyoes, B., Thukammee, W. and Pannangpetch, P. 2016. Protocatechuic acid restores vascular responses in rats with chronic diabetes induced by streptozotocin. *Phytotherapy Research* **30**: 227–233.

Semaming, Y., Kumfu, S., Pannangpetch, P., Chattipakorn, S.C. and Chattipakorn, N. 2014. Protocatechuic acid exerts a cardioprotective effect in type 1 diabetic rats. *Journal of Endocrinology* **223**: 13–23.

Senawong, T., Misuna, S., Khaopha, S., Nuchadomrong, S., Sawatsitang, P., Phaosiri, C., Surapaitoon, A. and Sripa, B. 2013. Histone deacetylase (HDAC) inhibitory and antiproliferative activities of phenolic-rich extracts derived from the rhizome of *Hydnophytum formicarum* Jack.: sinapinic acid acts as HDAC inhibitor. *BMC Complementary and Alternative Medicine* **13**: 232.

Setyaningsih, W., Saputro, I.E., Palma, M. and Barroso, C.G. 2015. Optimisation and validation of the microwave-assisted extraction of phenolic compounds from rice grains. *Food Chemistry* **169**: 141–149.

Shahidi, F. and Naczk, M. 1992. An overview of the phenolics of canola and rapeseed: chemical, sensory and nutritional significance. *Journal of the American Oil Chemists Society* **69**: 917–924.

Silambarasan, T., Manivannan, J., Priya, M.K., Suganya, N., Chatterjee, S. and Raja, B. 2015. Sinapic acid protects heart against ischemia/reperfusion injury and H9c2 cardiomyoblast cells against oxidative stress. *Biochemical and Biophysical Research Communications* **456**: 853–859.

Silambarasan, T., Manivannan, J., Raja, B. and Chaterjee, S. 2016. Prevention of cardiac dysfunction, kidney fibrosis and lipid metabolic alterations in L-NAME hypertensive rats by sinapic acid—Role of HMG-CoA reductase. *European Journal of Pharmacology* **777**: 113–123.

Song, Y., Cui, T., Xie, N., Zhang, X., Qian, Z. and Liu, J. 2014. Protocatechuic acid improves cognitive deficits and attenuates amyloid deposits, inflammatory response in aged AbetaPP/PS1 double transgenic mice. *International Immunopharmacology* **20**: 276–281.

Stalikas, C.D. 2007. Extraction, separation, and detection methods for phenolic acids and flavonoids. *Journal of Separation Science* **30**: 3268–3295.

Starmans, D. 1996. Extraction of secondary metabolites from plant material: a review. *Trends in Food Science & Technology* **7**: 191–196.

Stumvoll, M., Goldstein, B.J. and Van Haeften, T.W. 2005. Type 2 diabetes: principles of pathogenesis and therapy. *Lancet* **365**: 1333–1346.

Su, C.-Y., Ming, Q.-L., Rahman, K., Han, T. and Qin, L.-P. 2015. *Salvia miltiorrhiza*: traditional medicinal uses, chemistry, and pharmacology. *Chinese Journal of Natural Medicines* **13**: 163–182.

Szydłowska-Czerniak, A., Trokowski, K., Karlovits, G. and Szlyk, E. 2010. Determination of antioxidant capacity, phenolic acids, and fatty acid composition of rapeseed varieties. *Journal of Agricultural and Food Chemistry* **58**: 7502–7509.

Szydłowska-Czerniak, A. and Tułodziecka, A. 2014. Optimization of ultrasound-assisted extraction procedure to determine antioxi-

dant capacity of rapeseed cultivars. *Food Analytical Methods* **8**: 778–789.

Talagavadi, V., Rapisarda, P., Galvano, F., Pelicci, P. and Giorgio, M. 2016. Cyanidin-3-O-β-glucoside and protocatechuic acid activate AMPK/mTOR/S6K pathway and improve glucose homeostasis in mice. *Journal of Functional Foods* **21**: 338–348.

Tan, S.H., Mailer, R.J., Blanchard, C.L. and Agboola, S.O. 2011. Canola proteins for human consumption: extraction, profile, and functional properties. *Journal of Food Science* **76**: R16–R28.

Tanaka, T., Kawamori, T., Ohnishi, M., Okamoto, K., Mori, H. and Hara, A. 1994. Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis by dietary protocatechuic acid during initiation and postinitiation phases. *Cancer Research* **54**: 2359–2365.

Teh, S.-S. and Birch, E.J. 2014. Effect of ultrasonic treatment on the polyphenol content and antioxidant capacity of extract from defatted hemp, flax and canola seed cakes. *Ultrasonics Sonochemistry* **21**: 346–353.

Teh, S.-S., Niven, B.E., Bekhit, A.E.-D.A., Carne, A. and Birch, E.J. 2015. Microwave and pulsed electric field assisted extractions of polyphenols from defatted canola seed cake. *International Journal of Food Science & Technology* **50**: 1109–1115.

Tortorella, S.M., Royce, S.G., Licciardi, P.V. and Karagiannis, T.C. 2015. Dietary sulforaphane in cancer chemoprevention: the role of epigenetic regulation and HDAC inhibition. *Antioxidants and Redox Signalling* **22**: 1382–1424.

Townsend, N., Nichols, M., Scarborough, P. and Rayner, M. 2015. Cardiovascular disease in Europe — epidemiological update 2015. *European Heart Journal* **36**: 2696–2705.

Trachootham, D., Alexandre, J. and Huang, P. 2009. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nature Reviews Drug Discovery* **8**: 579–591.

Tsao, S.M., Hsia, T.C. and Yin, M.C. 2014. Protocatechuic acid inhibits lung cancer cells by modulating FAK, MAPK, and NF-kappaB pathways. *Nutrition and Cancer* **66**: 1331–1341.

Turrens, J.F. 2003. Mitochondrial formation of reactive oxygen species. *Journal of Physiology* **552**: 335–344.

Tzagoloff, A. 1963. Metabolism of sinapine in mustard plants. II. Purification & some properties of sinapine esterase. *Plant Physiology* **38**: 207–213.

Valdés, A., Vidal, L., Beltran, A., Canals, A. and Garrigos, M.C. 2015. Microwave-assisted extraction of phenolic compounds from almond skin byproducts (*Prunus amygdalus*): a multivariate analysis approach. *Journal of Agricultural and Food Chemistry* **63**: 5395–5402.

Velis, C.A. 2015. Circular economy and global secondary material supply chains. *Waste Management and Research* **33**: 389–391.

Vuorela, S. 2005. Analysis, isolation, and bioactivities of rapeseed phenolics. PhD dissertation, University of Helsinki.

Vuorela, S., Meyer, A.S. and Heinonen, M. 2003. Quantitative analysis of the main phenolics in rapeseed meal and oils processed differently using enzymatic hydrolysis and HPLC. *European Food Research and Technology* **217**: 517–523.

Wang, H.Y., Wang, H., Wang, J.H., Wang, Q., Ma, Q.F. and Chen, Y.Y. 2015a. Protocatechuic acid inhibits inflammatory responses in LPS-stimulated BV2 microglia via NF-kappaB and MAPKs signaling pathways. *Neurochemical Research* **40**: 1655–1660.

Wang, Y., Zhou, J., Fu, S., Wang, C. and Zhou, B. 2015b. Preventive effects of protocatechuic acid on LPS-induced inflammatory response in human gingival fibroblasts via activating PPAR-gamma. *Inflammation* **38**: 1080–1084.

Wen, D., Li, C., Di, H., Liao, Y. and Liu, H. 2005. A universal HPLC method for the determination of phenolic acids in compound herbal medicines. *Journal of Agricultural and Food Chemistry* **53**: 6624–6629.

Yamabe, N., Park, J.Y., Lee, S., Cho, E.-J., Lee, S., Kang, K.S., Hwang, G.S., Kim, S.-N., Kim, H.Y. and Shibamoto, T. 2015. Protective effects of protocatechuic acid against cisplatin-induced renal damage in rats. *Journal of Functional Foods* **19**: Part A 20–27.

Yang, Y., Duan, W., Lin, Y., Yi, W., Liang, Z., Yan, J., Wang, N., Deng, C., Zhang, S., Li, Y., Chen, W., Yu, S., Yi, D. and Jin, Z. 2013. SIRT1 activation by curcumin pretreatment attenuates mitochondrial oxidative damage induced by myocardial ischemia reperfusion injury. *Free Radical Biology and Medicine* **65**: 667–679.

Yin, M.C., Lin, C.C., Wu, H.C., Tsao, S.M. and Hsu, C.K. 2009. Apoptotic effects of protocatechuic acid in human breast, lung, liver, cervix, and prostate cancer cells: potential mechanisms of action. *Journal of Agricultural and Food Chemistry* **57**: 6468–6473.

Yu, X., Gouyo, T., Grimi, N., Bals, O. and Vorobiev, E. 2016. Pulsed electric field pretreatment of rapeseed green biomass (stems) to enhance pressing and extractives recovery. *Bioresource Technology* **199**: 194–201.

Yun, K.J., Koh, D.J., Kim, S.H., Park, S.J., Ryu, J.H., Kim, D.G., Lee, J.Y. and Lee, K.T. 2008. Anti-inflammatory effects of sinapic acid through the suppression of inducible nitric oxide synthase, cyclooxygase-2, and proinflammatory cytokines expressions via nuclear factor-kappaB inactivation. *Journal of Agricultural and Food Chemistry* **56**: 10265–10272.

Zhang, X., Shi, G.F., Liu, X.Z., An, L.J. and Guan, S. 2011. Anti-ageing effects of protocatechuic acid from Alpinia on spleen and liver antioxidative system of senescent mice. *Cell Biochemistry and Function* **29**: 342–347.

Zou, Y., Kim, A.R., Kim, J.E., Choi, J.S. and Chung, H.Y. 2002. Peroxynitrite scavenging activity of sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) isolated from *Brassica juncea*. *Journal of Agricultural and Food Chemistry* **50**: 5884–5890.