RESEARCH ARTICLE

An *In Vitro* Study Determining the anti-inflammatory activities of sinapinic acid-containing extracts generated from Irish rapeseed meal

Authors
Leah Quinn [1, 2, *], Kenneth R. Scott [3], Stephen P. Finn [1], Maria Hayes [2] and Steven G. Gray [1]

Affiliations
[1] Trinity Translational Medicine Institute, Trinity Centre for Health Sciences, St. James's Hospital, Dublin, Ireland
[2] Food BioSciences, Teagasc Food Research Centre, Ashtown, Dublin, Ireland
[3] Department of Pharmacology and Therapeutics, Trinity Centre for Health Sciences, St. James's Hospital, Dublin, Ireland
[4] Histopathology, Central Pathology Laboratory CPL30, St. James's Hospital, Dublin, Ireland

*** Correspondence:**
Leah Quinn, quinnl7@tcd.ie

Abstract

Sinapinic acid (SA) has been shown to possess various bioactive properties including anti-diabetic, anti-inflammatory and histone deacetylase inhibitory activities. However, the amount obtained from our diet is insufficient to produce beneficial effects on health. Therefore, isolating this bioactive phenolic from a natural source, such as rapeseed meal, could generate extracts containing concentrated amounts of SA which could be consumed as a functional food ingredient to prevent health-related disease, particularly inflammation.
Inflammation is a multi-faceted pathology, which plays a role in numerous diseases including cardiovascular disease, diabetes, arthritis and cancer. Current therapies such as non-steroidal anti-inflammatories (NSAIDs) are associated with various adverse side effects, with the result being an increase in research aiming to identify natural compounds which possess anti-inflammatory activity. In this work, an *in vitro* study assessed the anti-inflammatory activities of two sinapinic acid-containing extracts on human-derived peripheral blood mononuclear cells (PBMCs). Both extracts were found to significantly reduce the levels of key pro-inflammatory cytokines including TNF-alpha, IL-12 and IL-6. Importantly, these extracts were found to be more potent than commercial SA in terms of their anti-inflammatory activities. Results demonstrate the potential of these extracts as anti-inflammatory agents.

Keywords: inflammation, rapeseed, sinapinic acid, TNF-alpha, cytokine

Abbreviations:

ANOVA	Analysis of variance
CAGR	Compound Annual Growth Rate
COX	cyclo-oxygenase
GI	Gastrointestinal
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IV	Intravenous
LPS	Lipopolysaccharide
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
PA	Protocatechuic Aldehyde
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Protocatechuic acid
PRR	Pattern-recognition receptor
RA	Rheumatoid arthritis
SA	Sinapinic Acid
SEM	Standard error of the mean
TLR	Toll-like Receptor

1. Introduction

Inflammation can be defined as the immune systems response to harmful stimuli such as pathogens, damaged cells or toxic stimuli [1]. As such, inflammation is a defense mechanism necessary to prevent against infection and disease. However, if this process is uncontrolled, it can lead to acute, chronic inflammation which has serious effects on health. Diseases such as diabetes, cancer and osteoarthritis are linked to chronic, systemic inflammation [1]. The inflammatory process begins with recognition of harmful stimuli by 'Pattern-recognition receptors (PRRs)', which include Toll-like receptors (TLRs). Lipopolysaccharide (LPS) is a constituent in the outer wall of gram negative bacteria, and is recognized by TLR-4. Upon TLR-4 activation, transcription factors including NF-κB translocate to the nucleus where they stimulate the expression of pro-inflammatory cytokines [2].

Monocytes are one of the first groups 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. Cytokines can either be pro- or anti-inflammatory, which aides and inhibits 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 leads to tissue damage, organ failure and ultimately can prove fatal [1]. 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 [3]. Obesity is a key risk factor in the development of OA, with inflammatory cytokines the link between both conditions [4]. 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 [4]. These 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 [5]. Inhibiting these cytokines is a mechanism to treat inflammation and prevent development of associated diseases.

Current treatment 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 [6]. As previously mentioned, NSAIDs have adverse effects, particularly on the gastrointestinal system. Serious effects include intestinal bleeding, peptic ulcers and gastrointestinal (GI) perforation. Research is moving toward natural products as an alternative, in particular toward using naturally derived compounds that have anti-inflammatory activities with minimal side effects on the GI system [7].

Sinapinic acid (SA) has been shown to possess various bioactive properties including anti-diabetic, anti-inflammatory and histone deacetylase inhibitory activities[8-10]. In this brief research report, we describe the effects of the natural histone deacetylase inhibitor SA, on the release of pro-inflammatory cytokines from human peripheral blood mononuclear cells (PBMCs), demonstrating significant anti-inflammatory properties.

2. Materials and methods

2.1 Chemicals

All laboratory reagents were stored according to the manufacturer's instructions. All chemicals and reagents were of analytical standard and purchased from Merck (formerly Sigma Aldrich, Dublin, Ireland). These included: resazurin sodium salt, Histopaque®, RPMI-1640 media and Phosphate buffered saline.

2.2 Generation of sinapinic acid-containing extracts

Extract I which contained 0.053 mg/mL (5.3% w/v) SA and which was used during this work was generated and quantified as previously described in Quinn *et al*. [11].

Extract II containing 0.569 mg/ml (57% w/v) SA, was generated using a modified extraction protocol as follows:

The extraction of SA from rapeseed meal for Extract II was based on the method of Naczk et al [12], with modifications to the procedure as described. Sinapinic acid was extracted from the defatted meal by hydrolysing 100g with 800 mL of 4M NaOH for one hour. Three separate extractions were performed and pooled after hydrolysis. The hydrolysed samples were then centrifuged at 5000 rpm for 10 minutes, and the supernatants pooled. The supernatants were then acidified to below pH 2 using concentrated HCL (37%), before adding a half-volume of ethyl acetate and mixing. The mixture was then centrifuged at 5000 rpm for 5 min and the supernatants pooled. The mixture was then evaporated to dryness using rotary evaporation at 37°C.

A clean-up step was carried out with water in an extract: water ratio of 0.5 g: 35 mL (w/v). The extract was mixed using a rotating mixer (Benchmark Scientific, USA) for 10 min at room temperature, before sonication for 5 min. The extracts were then centrifuged at 5000 rpm for 10 min, and the supernatant containing the excess oil/ fat discarded. This process was repeated twice and the extracts were then allowed to dry overnight at 37°C.

2.3 Isolation of peripheral blood mononuclear cells (PBMCs)

Enriched buffy units were obtained from the Irish Blood Transfusion Service at St.

James's Hospital, Dublin 8, Ireland. The enriched buffy coat was diluted 1:1 (v: v) with RPMI-1640 media supplemented with 10 % FBS. A volume of 10 mL of Histopaque® (Merck, formerly Sigma Aldrich, Dublin, 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 centrifuged at 1650 rpm 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 volume was made up to 30 mL with complete media and centrifuged at 2000 rpm for 8 min with the brake on to wash the cells. This process was repeated once more, and the cell pellet was resuspended in a final volume of 45 mL of complete media.

2.4 Treatment of PBMCs with phenolic extracts and LPS

Extracts I, II and commercial SA were prepared at the relevant concentrations by dissolving in complete RPMI-1640 media.
Isolated peripheral blood mononuclear cells were pre-treated with the two extracts at various concentrations for 3 hours. Following this incubation period, LPS was then added at a concentration of 20 ng/mL for 2,4 and 6 hours. The supernatants were then collected and stored at -80°C until Enzyme-linked immunosorbent assay (ELISA) analysis was performed.

2.5 Resazurin assay

Cellular viability was determined using the resazurin assay [13]. Briefly, Resazurin sodium salt (formerly Sigma Aldrich, Dublin, Ireland) 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 color change was observed. Fluorescence was then measured at excitation / emission = 578 / 604 nm on a Fluoroskan ascent FL plate reader and analyzed using Ascent software (Thermo Fisher, USA).

2.6 ELISA assays

Measurement of TNF-alpha and Il-1β 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 ELISA kits from ImmunoTools (Friesoythe, 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-horseradish peroxidase (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 (Winooski, Vermont, U.S.A.). The data was then analyzed using KC Junior software (Biotek, USA).

2.7 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).

3. Results

3.1 Effects of extracts I and II on human-derived PBMC viability

Human PBMC's were isolated and seeded at a final concentration of 500,000 cells per well. Both extracts were prepared at the following concentrations 1, 0.5, 0.25, 0.125 and 0.062 mg/ml in media and added to each well. After 2, 4 and 6 hours, resazurin solution was added to each well and following incubation was 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 1). With extract II, only a concentration of 1 mg/mL significantly reduced PBMC viability at all three time-points ($P< 0.0001$), as shown in Figure 1.

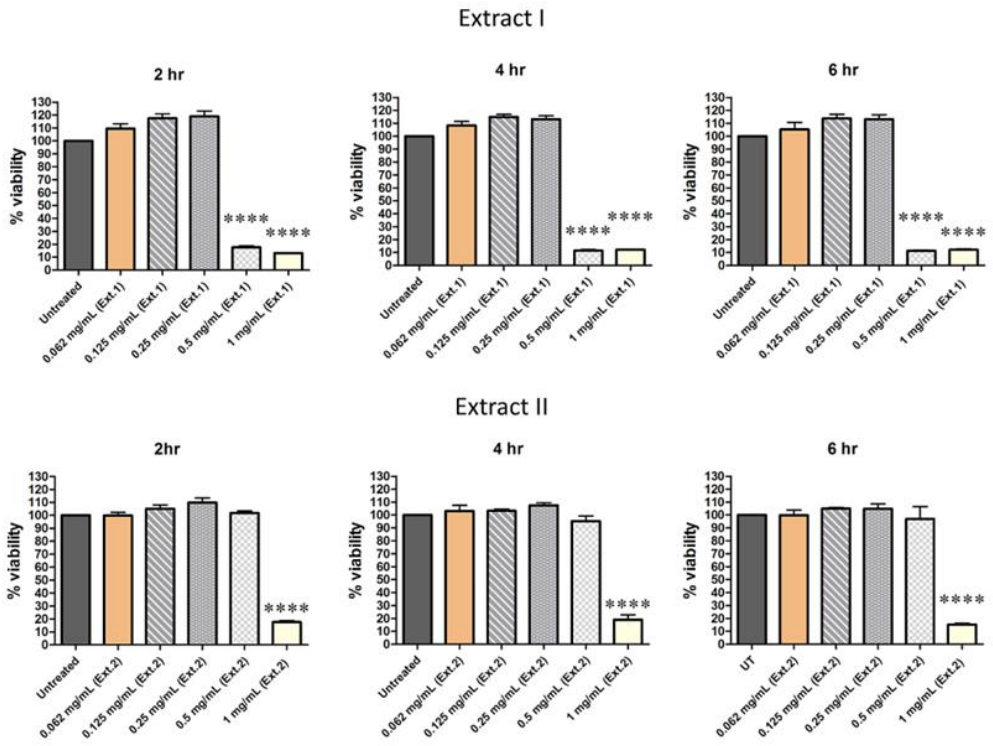

Figure 1. Effects of phenolic extract I and II on the viability of human PBMC's.
The resazurin assay was performed to determine cellular viability after 2, 4 and 6 hour treatments with extracts I and II. Statistical analysis was performed using a post-hoc Dunnett's Test vs untreated (UT) samples. Results are displayed as ± SEM, with statistical significance where $P < 0.05$. (N=3). **** $p< 0.0001$.

4.2 Phenolic extracts I and II significantly reduce the LPS-induced expression of TNF-alpha in human PBMCs

After 2 hours post-LPS addition, PBMCs treated with extract I at 0.25, 0.5 and 1 mg/mL had a significant reduction on TNF-alpha expression (Figure 2). 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 (Figure 2).

With extract II, PBMCS pre-treated with 0.062, 0.25, 0.5 and 1 mg/mL, resulted in significant reduction on TNF-alpha expression after 2 hours with LPS (Figure 2). Following a 6 hour incubation with LPS, PBMCs treated with extract II at concentrations of 0.125, 0.25, 0.5 and 1 mg/mL significantly reduced TNF-alpha expression (Figure 2).

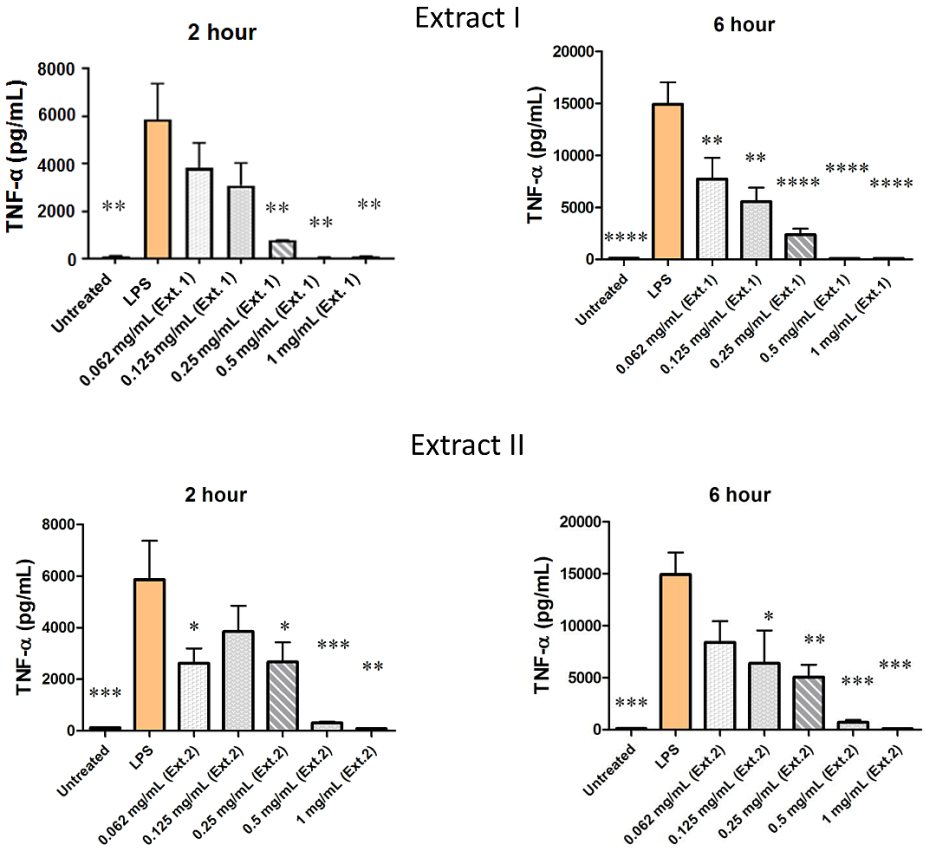

Figure 2. Effect of extracts I and II on LPS-induced expression of TNF-alpha in human PBMCs. Isolated PBMCs were pre-treated with both extracts at various concentrations for 3 hours. LPS (20 ng/mL) was then added for 2 and 6 hours before supernatants were collected for ELISA analysis. (A) 2h: PBMCs treated with extract 1 at 0.25, 0.5 and 1 mg/mL had a significant reduction on TNF-alpha expression. 6h: All concentrations of extract 1: 0.062, 0.125, 0.25, 0.5 and 1 mg/mL, significantly reduced TNF-alpha expression. (B) 2h: Extract II at 0.062, 0.25, 0.5 and 1 mg/mL significantly reduced on TNF-alpha expression. 6h: Extract II at concentrations of 0.125, 0.25, 0.5 and 1 mg/mL had significantly reduced TNF-alpha expression. Statistical analysis was performed using a Dunnett's Test vs LPS. Results are displayed as ± SEM (N=3). (* $p<0.05$; ** $p< 0.01$; *** $p< 0.001$; **** $p<0.0001$).

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

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 (Figure 3). After 4 hours, pre-treatment with extract I at 0.5 and 1 mg/mL significantly reduced IL-6 as shown in Figure 3. However, 6 hours post-LPS addition, all concentrations of extract I significantly reduced IL-6 expression (Figure 3). With extract II after 2 hours incubation with LPS

both 1 and 0.5 mg/mL significantly reduced IL-6 (Figure 3). Again, extract II at both 1 and 0.5 mg/mL significantly reduced IL-6 expression after 4 hours with LPS. 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 (Figure 3).

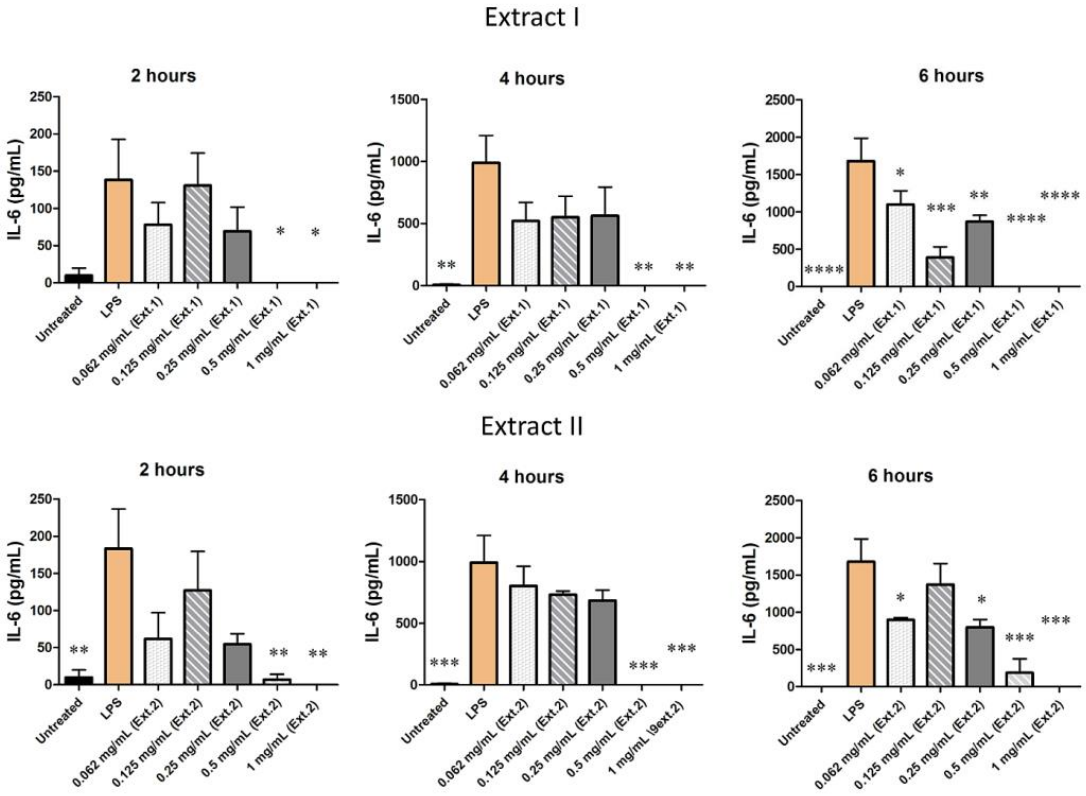

Figure 3. Effects of extracts I and II on LPS-induced expression of IL-6 in human PBMCs.
Isolated PBMCs were pre-treated with extracts I and II at various concentrations for 3 hours. LPS (20 ng/mL) was then added for 2, 4 and 6 hours before supernatants were collected for ELISA analysis. Extract I: 2h: Concentrations of 0.5 and 1 mg/mL significantly reduced IL-6 expression. 4h: Extract I at 0.5 and 1 mg/mL significantly reduced IL-6 expression. 6h: Extract I significantly reduced IL-6 expression at 0.062, 0.125, 0.25, 0.5 and 1 mg/mL. Extract II: 2h: Extract II at both 1 and 0.5 mg/mL significantly reduced IL-6 expression. 4h: At concentrations of 1 and 0.5 mg/mL, extract II significantly reduced IL-6 expression. 6h: Extract II significantly reduced IL-6 expression at 0.062, 0.25, 0.5 and 1 mg/mL. Statistical analysis was performed using a post-hoc Dunnett's Test vs LPS. Results are displayed as ± SEM (N=3). (* $p<0.05$; ** $p< 0.01$; *** $p< 0.001$; **** $p<0.0001$).

4.4 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 IL-12 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). At 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 (Figure 4). 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 (Figure 4).

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 (Figure 4). Following 6 hours with LPS, extract II significantly reduced IL-12 expression at concentrations of 1, 0.5 and 0.25 mg/mL (Figure 4).

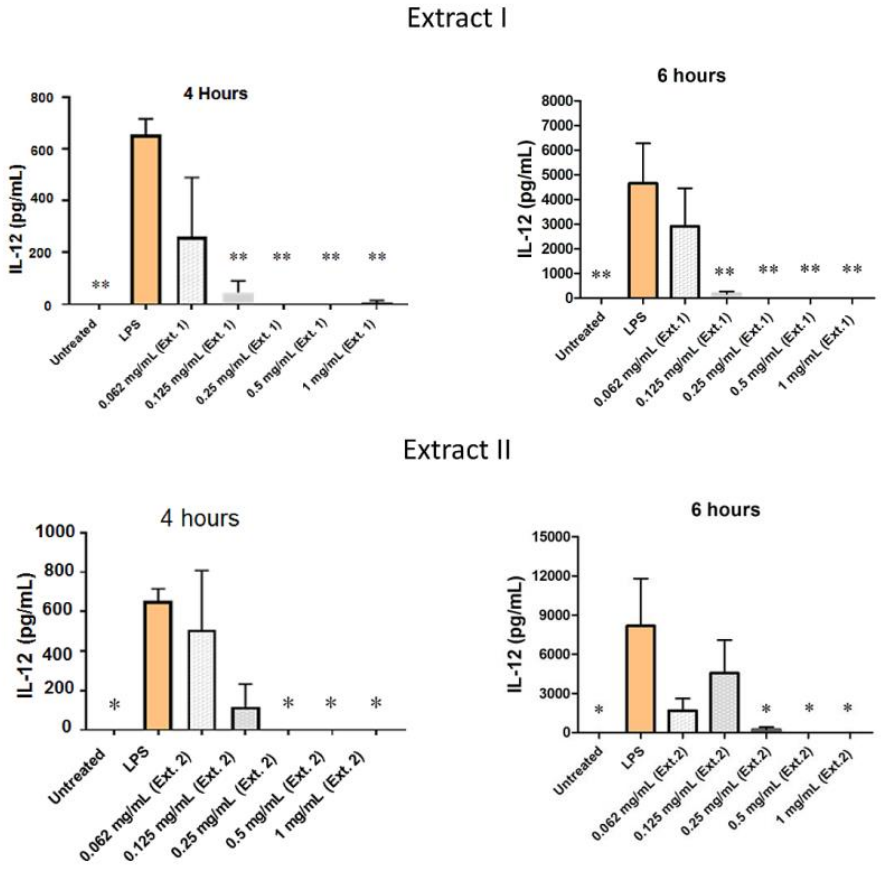

Figure 4. Effect of extracts I and II on LPS-induced expression of IL-12 in human PBMCs.
Isolated PBMCs were pre-treated with extracts 1 and II at various concentrations for 3 hours. LPS (20 ng/mL) was then added for 4 and 6 hours before supernatants were collected for ELISA analysis. Extract I: At 4h Extract I at concentrations of 1, 0.5, 0.25 and 0.125 mg/mL significantly reduced IL-12 expression. At 6h concentrations of 1, 0.5, 0.25 and 0.125 mg/mL, extract I significantly reduced IL-12 expression. Extract II: At 4h extract II at concentrations of1, 0.5 and 0.25 mg/mL significantly reduced IL-12 expression, and at 6h Extract II significantly reduced IL-12 expression at concentrations of 1, 0.5 and 0.25 mg/mL. Statistical analysis was performed using a Dunnett's Test vs LPS. Results are displayed as ± SEM (N=3). (* $p<0.05$; ** $p< 0.01$).

5. 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.

Many pro-inflammatory cytokines are regulated at the epigenetic level, particularly via histone deacetylases [14]. This has made them attractive for potentially targeting cancer [15], but also within the sphere of other pro-inflammatory conditions [16-18]. Natural products with epigenetic targeting activity therefore represent novel potential

therapeutic avenues to target inflammation, and are currently being investigated as alternatives to chemical drugs without the adverse side effects.

This work aimed to assess the anti-inflammatory activities of phenolic extracts containing sinapinic acid, which has been described as a natural histone deacetylase inhibitor[9].

PMBCs contain lymphocytes (T cells, B cells, NK cells) and monocytes. PBMCs are often used to study inflammation and are more representative of the *in-vivo* setting, as upon stimulation with LPS, monocytes secrete a variety of pro-inflammatory cytokines such as TNF-alpha and IL-12. The effects of SA containing extracts on PBMC viability were determined using the resazurin assay (Figure 1); therefore any reduction observed in cytokine levels can be attributed to the anti-inflammatory action of the extracts and not due to any adverse cytotoxicity. We found that Extracts I and II were able to significantly reduce the expression of key pro-inflammatory cytokines TNF-alpha, IL-6 and IL-12 in human-derived PBMCs (Figure 2-4). The results suggest that both the extracts and commercially derived sinapinic acid are able to inhibit the production of these cytokines.

Given that sinapinic acid is known as a histone deacetylase inhibitor it may be that these effects are due to changes at the epigenetic level in the regulation of expression of these cytokines. However, it must be noted that nutritional epigenetic regulators often have a multitude of additional effects. It may be that SA is functioning 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 [10, 19], and indeed it may be that it functions to alter the acetylation of NF-κB subunits affecting its nuclear translocation [20, 21].

The roles these cytokines play in disease have been well-established. 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 [7].

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 [22]. 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 [22].

Another form of arthritis, rheumatoid arthritis (RA), is also characterized by systemic inflammation and synovitis. Again, elevated levels of cytokines such as TNF-alpha and IL-6 are observed [23]. As such, these extracts have the potential to ameliorate common, debilitating auto-immune diseases.

SA containing rapeseed extracts I and II also offer advantages in terms of their side effects and delivery. The use of NSAIDs is associated with adverse side effects, commonly affecting the gastrointestinal system. One reason NSAIDs cause GI damage is due to inhibition of cyclo-oxygenase (COX) enzymes, which subsequently interferes with the efficacy of the mucous-bicarbonate barrier, negating its protective function [24]. 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 [24]. These extracts are generated from a natural by-product, rather than chemically synthesized, and so could offer anti-inflammatory activities without the harmful side-effects.

Although currently there are existing cytokine-targeting therapeutics, such as anti-TNF and IL-6 biologics, they are administered via intravenous (I.V) or subcutaneous injection [25].

Nutraceutical approaches to target pro-inflammatory milieus are a well investigated area. One example would be the Chinese traditional medicine (Danshen - Tasly Pharmaceuticals) [26], the first traditional Chinese Medicine to be cleared by the FDA for use in clinical trials. Danshen is traditionally used to treat cardiac (heart) and vascular (blood vessel) disorders such as atherosclerosis or blood clotting abnormalities[27]. In line with our data, Danshen has been shown to inhibit IL-6 production in macrophages[28,29].

Protocatechuic acid (PCA) and Protocatechuic aldehyde (PA) are phenolic acids found in many plants including green tea, rapeseed and Danshen. Both PA and PCA have been shown to affect the production of pro-inflammatory cytokines such as TNF-alpha and IL-6 under many experimental models including asthma, Alzheimer's, atherosclerosis, RA, and diabetes[30-36]. Other similar biologics such as curcumin [37-39], resveratrol [40], sulphoraphane [41] and Epigallocatechin-3-Gallate (EGCG) [42], have also well-established biological associations with the prevention or reduction of the secretion or activation of pro-inflammatory cytokine cascades [43].

The functional recovery of phenolics from agricultural by-product can obtain products such as SA that can be reinserted into the economy as new raw material [44]. In this regard, biologics, such as SA Extracts I and II could potentially be incorporated into food matrices, for example snacks or drinks, which are easily consumed and more patient-friendly. In this regard, plant stanol esters incorporated into food matrices such as Benecol® have shown clear clinical benefit in reducing cholesterol in individuals at risk of cardiovascular complications [45], and has resulted in FDA approval for incorporation into food matrices (FDA - 21 CFR 101.83) [46]. Such a strategy could be pursued for SA extracts. The nutraceutical market in Europe is predicted to register a Compound Annual Growth Rate (CAGR) of 7.5 % from 2019-2024 [47]. The reason for this increase is believed to be 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 [47]. Therefore developing a functional food product containing SA containing extracts could be well received in an established market.

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