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Inhibition of Proinflammatory Biomarkers in THP1 Macrophages by Polyphenols Derived From Chamomile, Meadowsweet and Willow bark

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Antiinflammatory compounds in the diet can alleviate excessive inflammation, a factor in the pathogenesis of common diseases such as rheumatoid arthritis, atherosclerosis and diabetes. This study examined three European herbs, chamomile (*Matricaria chamomilla*), meadowsweet (*Filipendula ulmaria* L.) and willow bark (*Salix alba* L.), which have been traditionally used to treat inflammation and their potential for use as antiinflammatory agents. Aqueous herbal extracts and isolated polyphenolic compounds (apigenin, quercetin and salicylic acid, 0–100 μM) were incubated with THP1 macrophages, and interleukin (IL)-1β, IL-6 and tumour necrosis factor-alpha (TNF-α) were measured. At concentrations of 10 μM, both apigenin and quercetin reduced IL-6 significantly ($p < 0.05$). Apigenin at 10 μM and quercetin at 25 μM reduced TNF-α significantly ($p < 0.05$). Amongst the herbal extracts, willow bark had the greatest antiinflammatory activity at reducing IL-6 and TNF-α production. This was followed by meadowsweet and then chamomile. The lowest effective antiinflammatory concentrations were noncytotoxic (MTT mitochondrial activity assay). The Comet assay, which was used to study the protective effect of the isolated phenols against oxidative damage, showed positive results for all three polyphenols. These are the first findings that demonstrate the antiinflammatory capacity of these herbal extracts.
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Keywords: chamomile; meadowsweet; willow bark; inflammation.

INTRODUCTION

It is acknowledged that polyphenols have antiinflammatory and antioxidant properties. For this reason, they may be considered as an alternative, natural approach to the treatment or management of inflammatory disease (Biasi *et al*., 2011). Recent studies have shown that consistent, low-grade inflammation contributes to the mechanism behind aging and age-related disease (Chung *et al*., 2009). Given our aging society, this is becoming an increasingly relevant public health issue.

In chronic systemic inflammation activated macrophages secrete proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), IL-1 (Comalada *et al*., 2006; Mamani-Matsuda *et al*., 2006; Mamani-Matsuda *et al*., 2004) and IL-6 (Shanmugam *et al*., 2008). Tumour necrosis factor-alpha and IL-1β are detectable in the synovial fluid of rheumatoid arthritis patients (Houssiau, 1995). The interest in alternative therapy options is increasing, because many do not want to be maintained on long-term pharmaceutical regimes (Astin, 1998).

Chamomile (*Matricaria chamomilla* L.), meadowsweet (*Filipendula ulmaria* L.) and willow bark (*Salix alba* L.) are medicinal herbs indigenous to Europe that have been used traditionally as antiinflammatory agents. Their health benefits in this regard may be due to their high levels of polyphenols, a large class of phytochemicals that are well known for health-promoting antioxidative (Trouillas *et al*., 2003) and antiinflammatory characteristics (Mueller *et al*., 2010; Manach *et al*., 2005).

Willow bark (*S. alba* L.) has some of the earliest historical citations to its antiinflammatory and analgesic properties (Vane, 2000), with salicin being the principal medicinal component and a precursor of acetylsalicylic acid (aspirin), a commonly used nonsteroidal antiinflammatory drug (NSAID) (Hedner and Everts, 1998). Meadowsweet has been shown to reduce complement activity and T-cell activation (Halkes *et al*., 1997; Blumenthal and American Botanical Council, 2000), both involved in the inflammatory response. In addition, chamomile has been shown to mitigate lipopolysaccharide (LPS)-induced COX-2 mRNA and protein expression in murine macrophages in a similar manner to NSAIDs (Srivastava *et al*., 2009). The principal antiinflammatory phenolic compounds in chamomile, meadowsweet and willow bark are apigenin, quercetin and salicylic acid, respectively (Harbourne *et al*., 2009b; Harbourne *et al*., 2009a; Harbourne *et al*., 2009c). Studies show that apigenin significantly reduced proinflammatory enzyme activity when cultured with murine macrophages by inhibiting mRNA expression of COX and iNOS (Garcia-Mediavilla *et al*., 2007; Liang *et al*., 1999). It has also been shown to inhibit IL-6 and IL-8 while simultaneously limiting the cytokine-induced expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin (Gerritsen, 1996). Most recently (Huang *et al*., 2010), apigenin has been shown to have a role in inhibiting the phosphorylation of inflammatory enzymes p35, MAP kinase and

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extracellular signal-regulated kinase with the result of suppressing the inflammation associated with late-stage allergic immune response. Quercetin was shown to decrease NO and TNF-α production in human macrophages, as well as down-regulate COX-2 and iNOS- mRNA. Salicylic acid incubation with peritoneal macrophages was associated with a slight decrease in COX-1 and COX-2 enzyme activity (Tordjman *et al*., 1995).

The aim of this study was to assess the antiinflammatory activity of three herbs and their principal polyphenolic constituents using cytokines, IL-1β, IL-6 and TNF-α, as biomarkers of inflammation in the macrophage cell model associated with inflammation. The ability of the polyphenols to protect against hydrogen peroxide (H_2O_2)-induced oxidative damage was also measured. The combination of herbs under study is novel, and as such, this investigation is the first to analyse their particular antiinflammatory and cytotoxicity properties.

MATERIALS AND METHODS

Preparation and quantification of plant extracts. Using a modified method of that described by Harbourne *et al*. (2009a, 2009b), phenolic compounds were extracted from chamomile (*M. chamomilla* L.), meadowsweet (*F. ulmaria* L.) and willow bark (*S. alba*). Briefly, dried meadowsweet, willow bark and chamomile (flowers) were purchased from The Organic Herb Trading Company (Somerset, UK). Chamomile (7.5 g) was extracted in 100 mL water at 90 °C for 20 min. Dried aerial parts of meadowsweet and willow bark (7.5 g) were extracted in 100 mL water at 90 °C for 15 min. Plant extracts were sterile filtered through nalgene polyethersulfone (90 mm diameter, 0.2 μm pore size) units. The total phenolic content of extracts was measured using the Folin–Ciocalteu method of Singleton and Rossi (1965), as described by Harbourne *et al*. (2009a, 2009b, 2009c). Polyphenols were also utilised in isolated form; quercetin dehydrate and apigenin were both purchased from Extrasynthèse (Genay, France), whereas salicylic acid was bought from Sigma Aldrich, Ireland. These compounds will be herein referred to as *isolated* to distinguish them from herbal extracts.

Cell lines and culture protocol. Initially, cells were treated with isolated phenols apigenin, quercetin and salicylic acid to establish concentration–response data. Once these data were obtained, cells were treated in the same manner using plant extracts containing the aforementioned phenols.

THP1 cells (human monocytic leukemic cell line) were purchased from the ECACC (European Animal and Cell Collection). Human B lymphocytes were purchased from NIGMS Human Genetic Cell Repository (Coriell, California, USA). Cells were maintained using RPMI 1640 (Sigma Aldrich, Ireland), supplemented with 2 nM L-glutamine, 10% heat-inactivated foetal bovine serum (FBS) and 1% penicillin–streptomycin (10 000 units of penicillin and 10 mg of streptomycin per millilitre) (all Sigma Aldrich, Ireland). Cells were kept viable in a humidified atmosphere, at 37 °C, 5% CO_2.

THP1 differentiation, treatment and stimulation. On Day 0, the THP1 monocytes were seeded at 4×10^5/mL in 2 mL 24-well plates and treated with phorbol-12-myristate-13-acetate (Sigma Aldrich, Ireland) at 100 ng/mL. After 72 h, medium was replaced with FBS-free RPMI 1640 for another 24 h. Medium was refreshed, and the cells were treated with herbal extract or the isolated polyphenols. Quercetin dihydrate, apigenin and salicylic acid were delivered to cells using DMSO as the vehicle. The final concentration in media ranged from 0 to 100 μM. Stock solutions of isolated phenolic compounds were stored in DMSO at −20 °C. Where DMSO was used as the vehicle, it did not exceed 0.5% (v/v) of 2 mL media volume. Regarding sterile filtered plant extracts, either a 10 or 50 μL aliquot was added to culture wells. The treatment period in all cases was 24 h. Following this, LPS from *Escherichia coli* O127:B8 (Sigma Aldrich, Ireland) was added at 1 μg/mL for a further 24 h. Subsequently, the culture liquid was taken up from each well and centrifuged at 1800 rpm for 5 min (Hettich Zentrifugen, Rotina 38 R, rotor model 1724). The supernatants were stored at −80 °C until analysed.

Determination of IL-1β, IL-6 and TNF-α in culture media. Cytokine analysis with quantitative sandwich enzyme-linked immunosorbent assay A (Human Quantikine kits, R&D Systems, Abingdon, UK) was employed to test for human IL-1β, IL-6 and TNF-α. Cell culture supernatants, preserved at −80 °C, were thawed and analysed immediately. The procedure was conducted in accordance with manufacturer's instructions. This reaction was stopped using 2 N sulphuric acid, and the plate was read at 450 nm.

Cell viability assays. The principal viability assessment technique employed was 3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide (MTT). Cells were cultured as normal in a 96-well plate in phenol red-free medium (Sigma Aldrich, Wicklow). The MTT solution was applied at 10% of the cell suspension volume in each well, and the plate was incubated at 37 °C + 5% CO_2 for 3–4 h until MTT formazan crystals were visible in the culture liquid. MTT solvent (0.1 N HCl in anhydrous isopropanol) was applied directly to the MTT reagent, and the plate was shook until complete dissolution of the formazan crystals. The plate was read at 570 nm.

Genotoxicity assay (Comet assay (single cell gel electrophoresis)). Human B lymphocytes (Coriell Cell Repositories, New Jersey, USA) were employed for the operation of the Comet assay. The method of Singh *et al*. (1988) was adapted to incorporate various modifications described by Rojas *et al*. (1999). B cells were incubated for 24 h with varying concentrations (0–50 μM) of the polyphenol of interest. Following this, samples were treated with 19 μM H_2O_2 to induce oxidative damage, and control cells were treated with polyphenols but left unexposed to H_2O_2. Unpolished glass slides (Corning, NL, Mexico), previously coated with two layers of 1.5% NMPA (90 μL), were submerged in lysis buffer for 1 h, chilled to 4 °C. The electrophoresis tank was filled with chilled buffer, and the system was run for 20 min at 25 V and 300 mA, before submerging in neutralisation buffer for 15 min. Slides were dried overnight and stained the following day with 40 μL Dapi staining solution. They were visualised under a Leica DMLB fluorescent

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compound microscope with digital imaging software (CometScore Version 1.5).

Statistical analysis. Data were presented as the mean ± standard deviation of duplicate samples run over three independent experimental assays for each parameter. Statistical analysis was conducted using the PASW Statistics 18 software (formerly SPSS Inc.). Data were analysed by analysis of variance with Tukey's *post hoc* test with a confidence interval of 95%.

RESULTS

Cell viability assays

An MTT assay was carried out to determine the viability of the THP1 population (Fig. 1A) in response to 24 h incubation with 0–100 µM of quercetin, apigenin or salicylic acid as well as herbal extracts at the low and high concentration range. In addition, human B lymphocytes (Fig. 1B) were assessed for viability in the presence of the three polyphenols at 10, 50 and 100 µM. Isolated phenols were universally noncytotoxic until a level of 10 µM ($p > 0.05$). At a concentration of 25 µM of apigenin and quercetin, the viability was reduced significantly (both $p < 0.05$). Although salicylic acid had no significant effect on viable numbers even at 100 µM. For the most part, it was considered that concentrations above 50 µM were excessive and were excluded from experiments herein. The aqueous extracts showed no cytotoxicity, with population numbers remaining steady at low and high concentrations.

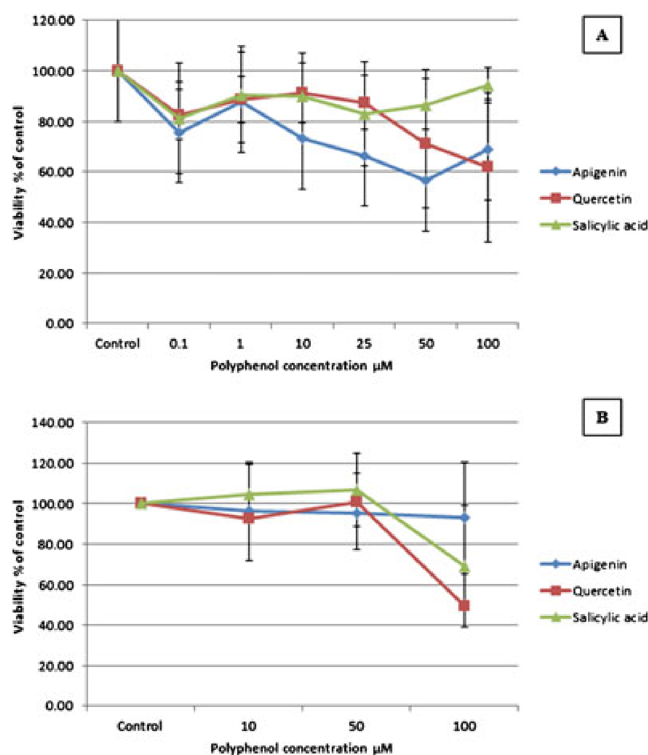

Figure 1. The viability of (A) THP1 macrophages and (B) human B lymphocytes, as assessed by an MTT-based cytotoxicity method (after incubation with isolated apigenin, quercetin and salicylic acid for 24 h). Error bars represent standard deviation, which is representative of three independent experiments. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

THP1 macrophage cytokine production in response to isolated polyphenols

Apigenin, quercetin and salicylic acid were applied to cells at concentrations ranging 0–50 µM. Apigenin induced a concentration–response reduction of the cytokines TNF-α and IL-6 (Table 1). At 1 µM, apigenin significantly lowered both of these cytokines ($p < 0.05$). Apigenin did not induce a concentration–response pattern for IL-1β, but at 25 or 50 µM a reduction of 31% occurred ($p < 0.05$). Quercetin acted in a similar pattern to that of apigenin, reducing TNF-α and IL-6 in a concentration-dependent manner (Fig. 2). At all concentrations, it resulted in a significant reduction in IL-6, and at 25 µM it produced a significant reduction in TNF-α ($p < 0.05$). For IL-1β, no significant reductions were obtained. Salicylic acid significantly ($p < 0.05$) lowered IL-6 at all concentrations (Table 1). It had no significant effect on either TNF-α or IL-1β, showing a random pattern of reductions ($p > 0.05$).

Comparison of the response of the polyphenols showed that at 10 µM both quercetin and apigenin were significantly more effective inhibitors of IL-6 than salicylic acid ($p < 0.05$). At this concentration, quercetin and apigenin were equally effective, with no significant difference between their mean reductions of IL-6 ($p > 0.05$). Tumour necrosis factor-alpha was reduced more effectively by 10 µM apigenin than by quercetin or salicylic acid ($p < 0.05$). In reducing IL-1β, apigenin was the only phenol to significantly inhibit production (Table 1); this was at concentrations upwards of and including 25 µM ($p < 0.05$). At concentrations 25 and 50 µM, apigenin and quercetin (Fig. 2) were more effective than was salicylic acid at lowering IL-6 and TNF-α ($p < 0.05$). At 50 µM, apigenin and quercetin were able to reduce all cytokines with the same efficacy ($p < 0.05$).

THP1 macrophage cytokine production in response to plant extracts

An aliquot of 10 or 50 µL of willow, chamomile or meadowsweet extract was applied to the culture well resulting in phenol concentrations of 0–4 µM or 7–20 µM (Table 2). At the lower range of concentrations, willow bark extract (containing 1.4 µM salicylic acid) induced the greatest change in cytokines TNF-α and IL-6, with an average decrease of 54% and 38%, respectively ($p < 0.05$) (Table 3). Willow bark extract's effect on IL-1β was comparatively lower, achieving a 15.6% reduction, which was not significant ($p < 0.05$) (Table 2). Meadowsweet (containing 3 µM quercetin) significantly reduced TNF-α and IL-1β by 33.7% and 45.4% ($p < 0.05$), respectively. Its reduction of IL-6 was not significant at 16.3% ($p > 0.05$) (Table 3). Chamomile (containing 4 µM apigenin) reduced IL-6 by 22%, TNF-α by 5.7% and IL-1β by 18.7% ($p > 0.05$). When the concentration was increased fivefold to a 50 µL application, all three extracts achieved significant reduction in IL-6 concentration (Table 3). These changes were significantly greater than those at the low concentration ($p < 0.05$). At this higher concentration, chamomile did not have a significantly different effect on TNF-α or IL-1β ($p > 0.05$) (Table 3).

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Table 1. The effects of isolated polyphenols on proinflammatory cytokine production in LPS-stimulated THP1 macrophages

	Dose (μM)	TNF-α	±SD	Δ change	p	IL-6	±SD	Δ change	p	IL-1β	±SD	Δ change	p
Apigenin	0	100	0			100	0			100	0		
	0.1	133	16	−33	0.06	93	31	7	0.99	63	11	37	0.26
	1	146	28	−46	0.00	68	21	32	0.02	62	4	38	0.24
	10	23	17	77	0.00	7	3	93	0.00	74	12	26	0.63
	25	14	5	86	0.00	3	3	97	0.00	69	27	31	0.08
	50	11	4	89	0.00	3	3	97	0.00	69	18	31	0.01
Quercetin	0	100	0			100	0			100	0		
	0.1	106	2	−6	0.76	65	11	35	0.00	62	13	38	0.28
	1	107	1	−7	0.62	58	15	42	0.00	79	6	21	0.83
	10	80	4	20	0.97	7	1	93	0.00	70	23	30	0.54
	25	40	10	60	0.00	13	8	88	0.00	61	13	39	0.27
	50	16	2	84	0.00	4	1	96	0.00	79	23	21	0.83
Salicylic acid	0	100	0			100	0			100	0		
	0.1	94	6	6	0.88	48	4	52	0.01	79	15	21	0.15
	1	99	8	1	1.00	61	4	39	0.04	96	7	4	1.00
	10	105	6	−5	9.54	64	22	36	0.07	98	4	2	1.00
	25	99	5	1	1.00	41	7	59	0.00	92	3	8	0.90
	50	100	1	0	1.00	36	7	64	0.00	79	8	21	0.15

Cytokine levels are expressed as a percentage of controls. Controls were LPS-stimulated macrophages which did not receive phenol or vehicle treatment. *p* value indicates significant difference between dose and control ($p < 0.05$). Standard deviation was calculated based on data from three independent experiments. LPS, lipopolysaccharide; TNF-α, tumour necrosis factor-alpha; SD, standard deviation from the mean; IL-6, interleukin-6; IL-1β, interleukin-1 beta; Δ change, the difference between the cytokine production of the control and that of the sample.

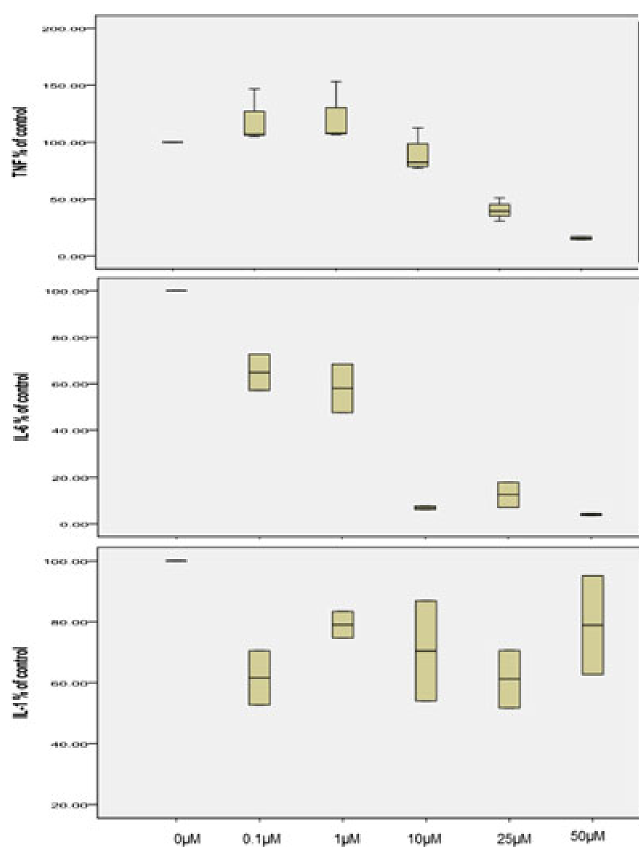

Figure 2. THP1 macrophage production of (A) TNF-α, (B) IL-6 and (C) IL-1β following incubation with 0–100 μM quercetin for 24 h. Error bars represent standard deviation of three separate experiments. An asterisk indicates statistically significant difference ($p < 0.05$), where samples were compared with the control in each case.

However, meadowsweet and willow bark brought about significantly greater reductions at this concentration range for all three cytokines ($p < 0.05$).

Comet assay of THP1 macrophages after incubation with isolated polyphenols

The Comet assay, or single-cell gel electrophoresis method, was employed to determine the ability of the phenols to protect against oxidation induced by exposure of cells to hydrogen peroxide (H_2O_2). Human B lymphocytes were utilised because their nature as a suspension cell line was more suitable for the protocol than an adherent line such as the THP1 macrophages.

Negative control cells, left untreated with polyphenols or H_2O_2 (0 μM–), remained intact, showing minimal DNA damage with a mean Comet score of 0.5 (Fig. 3). Positive control cells (0 μM +), which were assaulted with H_2O_2, showed DNA damage with a mean Comet score of 3.2, significantly different from negative controls ($p < 0.001$). When cells were incubated with phenols at the highest concentration (50 μM) and shocked with H_2O_2, the mean Comet score was raised above 3 (Fig. 2), which was significantly different from the negative control ($p < 0.05$).

After incubation with 10 μM apigenin or quercetin and exposure to H_2O_2, cells showed little DNA damage (Fig. 3A and B), with no significant difference from the negative control ($p > 0.05$). Salicylic acid at 10 μM induced a mean Comet score of 1.75 (Fig 3C), which was significantly greater than that of the negative control ($p < 0.05$). At 1 μM, no polyphenols induced significant DNA damage ($p > 0.05$). Cells treated with polyphenols and without a H_2O_2 challenge showed no

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Table 2. Concentration of polyphenols apigenin, quercetin and salicylic acid in extracts of chamomile, meadowsweet and willow bark

	Herbal extract (7.5 g/100 mL water)				Phenol concentration in cell media at 10 μL (low dose range)			Phenol concentration in cell media at 50 μL (high dose range)		
Herb	Total phenols (g/L)	Apigenin (g/L)	Quercetin (g/L)	Salicylic acid (g/L)	Apigenin (μM)	Quercetin (μM)	Salicylic acid (μM)	Apigenin (μM)	Quercetin (μM)	Salicylic acid (μM)
Chamomile	1.68	0.22			4			20		
Meadowsweet	10.07		0.14	0.01		3			15	
Willow bark	7.74			0.02			1.4			7

Herbs were extracted at a ratio of 7.5 g/100 mL water with phenol concentrations determined using the total phenol method of Singleton and Rossi (1965), modified and described by Harbourne *et al.* (2009b, 2009c). Plant extracts were sterile filtered and applied to cells in 24-well plates at volumes of 10 and 50 μL resulting in low and high doses. Standard deviation was calculated based on data from three independent experiments.

Table 3. Cytokine production in THP1 macrophages after incubation for 24 h with sterile-filtered plant extracts containing polyphenols at a low and high dose range

Extract	Phenol dose	Control	IL-6	SD	Δ change	*p*	TNF-α	SD	Δ change	*P*	IL-1β	SD	Δ change	*p*
Low dose range														
Chamomile	4 μM apigenin	100	76	12	25	0.10	94	5	6	0.95	81	0	19	0.29
Meadowsweet	3 μM quercetin	100	84	11	16	0.50	66	11	34	0.01	55	15	45	0.01
Willow bark	1.4 μM salicylic acid	100	62	7	38	0.02	46	10	54	0.00	84	12	16	0.45
High dose range														
Chamomile	20 μM apigenin	100	40	8	60	0.00	95	0.2	5	0.98	67	5.0	33	0.31
Meadowsweet	15 μM quercetin	100	48	1	52	0.00	61	0.0	39	0.00	49	0.7	51	0.00
Willow bark	7 μM salicylic acid	100	4	0	96	0.00	2	0.1	98	0.00	18	1.4	82	0.00

Low and high doses were prepared by addition of either 10 or 50 μL of aqueous extract. Low and high doses provided apigenin from chamomile at 4 and 20 μM, quercetin from meadowsweet at 3 and 15 μM and salicylic acid from willow bark at 1.4 and 7 μM, respectively. Data were expressed as percentage of control, where control samples were stimulated with LPS but received no polyphenol treatment. *p* value indicates significant difference between dose and control ($p < 0.05$). Standard deviation was calculated based on data from three independent experiments. LPS, lipopolysaccharide from *E. coli* O127:B8 applied to cell culture media at 1 μg/mL; IL-6, interleukin 6; TNF-α, tumour necrosis factor-alpha; IL-1β, interleukin-1 beta; Δ change, the difference between the cytokine production of the control and that of the sample; SD, standard deviation from the mean.

significant damage up to concentrations of 10 μM. At 50 μM, significant damage was induced ($p < 0.05$).

The cells were visualised using a Leica DMLB fluorescent microscope, and the images were digitally recorded. Two different treatment results are shown (Fig. 4). Point A shows an intact, control cell with a mean Comet score of 0.5. At Point B, the trail of excised DNA is visible after the cell was incubated with 50 μM quercetin (+ H_2O_2), gaining a Comet score of 3.6.

DISCUSSION

Many studies have reported antiinflammatory effects of these phenols, using only isolated compounds rather than plant extracts, as was done in this instance. Here, a significant reduction in proinflammatory cytokines IL-6 and TNF-α was demonstrated in response to quercetin. These findings are consistent with those reported by Rao *et al.* (2005), who achieved a 40% reduction in TNF-α amongst murine peritoneal macrophages treated with quercetin. Similarly, Comalada *et al.* reported 76% inhibition of TNF-α after 25 μM quercetin upon incubating murine macrophages (Comalada *et al.*, 2006).

Apigenin, from 10 μM onward, demonstrated good antiinflammatory activity in reducing IL-6 and TNF-α by around 70%–90% (Table 3) ($p < 0.05$). Shanmugam *et al.* (2008) described a similar reduction in TNF-α of around 60% between 10 and 20 μM apigenin incubated with murine macrophages. Interleukin-1β was not reduced in a consistent manner by any polyphenol, but apigenin, from 25 to 50 μM, was effective.

Salicylic acid induced the lowest antiinflammatory response of the compounds tested. A significant reduction of 60% was induced in IL-6 at 25 μM ($p < 0.001$), but this is a higher dosage than was required to reach significance for other phenols. Because this phenol is related to acetylsalicylic acid (aspirin), a well-established antiinflammatory drug (Preston *et al.*, 1989), these results were surprising. Aspirin works by inhibiting the cyclooxygenase enzymes (COX-1 and COX-2) (Hinz *et al.*, 2000), but salicylate operates through inhibition of transcription factor NF-κB (Kopp and Ghosh, 1994). Salicylate has been shown to be less effective *in vitro* than *in vivo*, where it demonstrates excellent antiinflammatory ability on a par with aspirin (Preston *et al.*, 1989). Therefore, the effects may be more successful in an animal model.

Unique to this study was the examination of herbal extracts. These herbs, ideal for use in a complementary medicinal product, produced similarly effective reduction in inflammation to the commercially isolated phenolic compounds. The results demonstrate that the herbal extracts did have antiinflammatory properties *in vitro*, although to a lesser extent, to isolated polyphenols. Willow bark extract caused the greatest reduction of TNF-α and IL-6, which were down-regulated by 54.5% and 38% at the low concentration range, and when the concentration was increased, this reduction grew to 98% and 96%, respectively (Table 3). This

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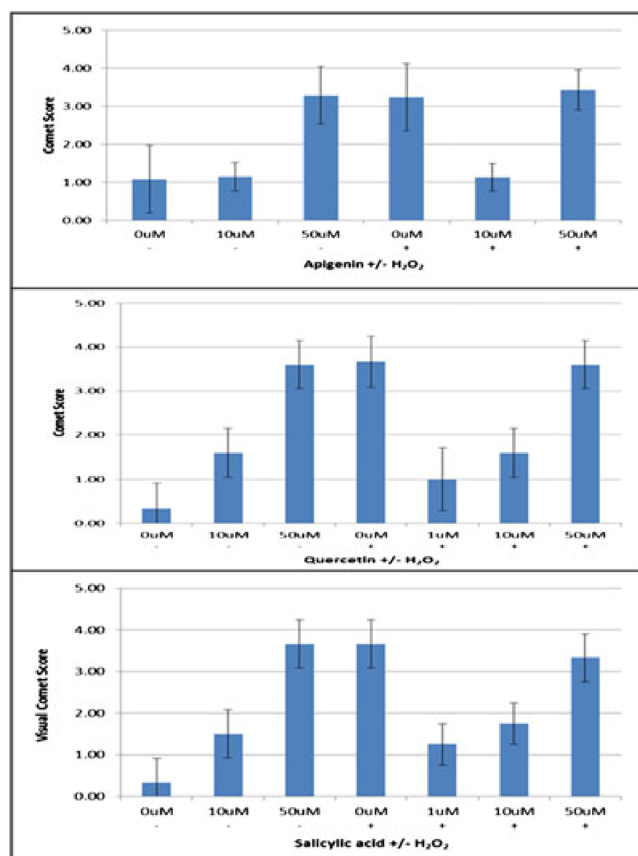

Figure 3. Comet assay to assess the protective effect of apigenin, quercetin and salicylic acid against H_2O_2-induced oxidation. Cells incubated at doses 0–100 μM for 24 h. Error bars represent standard deviation of three separate experiments. An asterisk indicates statistically significant difference ($p < 0.05$), where samples were compared with the negative control in each case. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

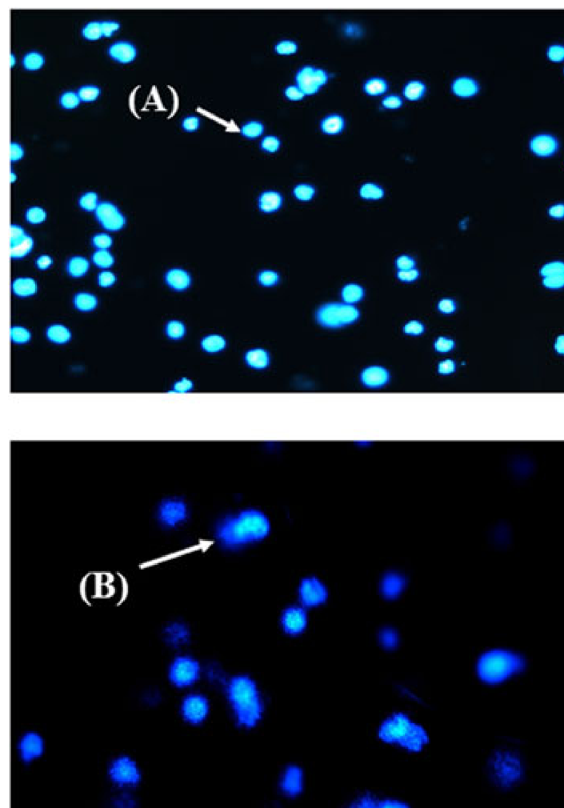

Figure 4. B cells stained with 40 μL DAPI after Comet assay. (A) A group of intact cells; note the absence of any stray DNA material. (B) A cell with moderate DNA strand breaks as can be seen as the nucleic material leaks from the cell after incubation with 50 μM quercetin. Digital image captured at ×20 magnification in a Leica digital imaging microscope. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

increase in concentration helped willow bark reduce IL-1β by 82%, having failed to reach significance at the low concentration. Data published by Harbourne *et al.* (2009a, 2009c) showed that, although willow bark extract had a lower total phenol concentration, it had a higher flavonoid content than that of meadowsweet. Moreover, it has a high salicin content, which is a known antiinflammatory agent (Vane, 2000). Meadowsweet extract was effective at low and high concentrations, except in the case of IL-6, which, at the low concentration, did not significantly affect production. Chamomile was the least potent antiinflammatory. It only reached significance at the high concentration for IL-6 (Table 3). Chamomile's total phenol concentration was the lowest amongst the three herbs at 0.5 g/L (Table 3).

These are the first data to indicate successful antiinflammatory activity in these herbal extracts.

Interleukin-1β behaves differently from the TNF family in that it binds to two types of cell-surface receptor (IL-1RI and IL-1RII) (Symons *et al.*, 1995; Janeway, 2005). Soluble IL-1β receptors compete with cell-bound receptors. Upon release of these soluble receptors from the cell, they can bind to IL-1β precursor and prevent development of the peptide to mature status (Symons *et al.*, 1995). The mixed results for IL-1β seen in the present study may be related to the different binding abilities of certain cells. Although macrophages are producers of the cytokine, evidence suggests that only monocytes bear the receptors. If this was the case, it would imply that there is no autocrine effect occurring and that IL-1β signalling in an isolated macrophage cell line would be limited. At very high concentrations, apigenin and quercetin achieved significant reductions in IL-1β, but results at lower concentrations and for salicylic acid were inconclusive. Interleukin-1β is heavily indicated in the production of IL-6 (Janeway, 2005).

At concentrations of 10 μM of apigenin or quercetin, 80%–90% of the control population remained viable. The herbal extracts had no cytotoxic effect on the cell population at either the low or high concentration ranges, thus validating their use as antiinflammatory agents. The Comet assay, conducted on B lymphocytes incubated with isolated phenols, supported the phenolic compound's antioxidant profile by demonstrating a protection against induced oxidative DNA strand breaks. At 1 and 10 μM, the polyphenols prevented strand breaks. Polyphenols alone did not induce DNA damage until a concentration of 50 μM, at which stage they were genotoxic and had lost their protective antioxidant effect. This was consistent with results found by Lautraite *et al.* (2002), where quercetin was tested on the Chinese hamster cell line V79. The inhibitory effect of lower concentrations of quercetin was likely linked to its impact on enzymes such as cytochrome P450 (CYP450) and CYP1A2, which are involved in the metabolism of known carcinogens. Here, we showed similar results but using a human lymphocyte line. It is incumbent upon the

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authors to note that the aqueous extracts would have been a relevant factor in the Comet experiments. This is something for consideration in future work.

This study has, for the first time, shown that extracts from the medicinal herbs chamomile, meadowsweet and willow bark, in which apigenin, quercetin and salicylic acid are amongst the main polyphenols, have positive antiinflammatory activity in a human macrophage population. These compounds were also shown to be noncytotoxic and to have protective antioxidant effects. These findings would justify a move toward an *in vivo* study of the herbs and their bioactive compounds.

Conflict of Interest

The authors declare no conflict of interest.

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