Journal of Inflammation Research

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ORIGINAL RESEARCH

Effect of plant extracts on H_2O_2-induced inflammatory gene expression in macrophages

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This article was published in the following Dove Press journal:
Journal of Inflammation Research
24 June 2014
Number of times this article has been viewed

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Background: *Arctium lappa* (AL), *Camellia sinensis* (CS), *Echinacea angustifolia*, *Eleutherococcus senticosus*, *Panax ginseng* (PG), and *Vaccinium myrtillus* (VM) are plants traditionally used in many herbal formulations for the treatment of various conditions. Although they are well known and already studied for their anti-inflammatory properties, their effects on H_2O_2-stimulated macrophages are a novel area of study.

Materials and methods: Cell viability was tested after treatment with increasing doses of H_2O_2 and/or plant extracts at different times of incubation to identify the optimal experimental conditions. The messenger (m)RNA expression of *TNFα*, *COX2*, *IL1β*, *NFκB1*, *NFκB2*, *NOS2*, *NFE2L2*, and *PPARγ* was analyzed in macrophages under H_2O_2 stimulation. The same genes were also quantified after plant extract treatment on cells pre-stimulated with H_2O_2.

Results: A noncytotoxic dose (200 μM) of H_2O_2 induced active mRNA expression of *COX2, IL1β, NFE2L2, NFκB1, NFκB2, NOS2*, and *TNFα*, while *PPARγ* was depressed. The expression of all genes tested was significantly ($P<0.001$) regulated by plant extracts after pre-stimulation with H_2O_2. *COX2* was downregulated by AL, PG, and VM. All extracts depressed *IL1β* expression, but upregulated *NFE2L2. NFκB1, NFκB2*, and *TNFα* were downregulated by AL, CS, PG, and VM. *NOS2* was inhibited by CS, PG, and VM. *PPARγ* was decreased only after treatment with *E. angustifolia* and *E. senticosus*.

Conclusion: The results of the present study indicate that the stimulation of H_2O_2 on RAW267.4 cells induced the transcription of proinflammatory mediators, showing that this could be an applicable system by which to activate macrophages. Plant extracts from AL, CS, PG, and VM possess in vitro anti-inflammatory activity on H_2O_2-stimulated macrophages by modulating key inflammation mediators. Further in vitro and in vivo investigation into molecular mechanisms modulated by herbal extracts should be undertaken to shed light on the development of novel modulating therapeutic strategies.

Keywords: inflammation, qRT-PCR, RAW264.7 cells, nutraceuticals

Introduction

The use of herbal/botanical products worldwide enjoys increasing popularity.[1,2] It is known that the great variety of plant secondary metabolites are excellent sources for components such as polyphenols, which exhibit antioxidant, anticarcinogenic, antimutagenic, and anti-inflammatory effects.[3–5] The understanding of the cellular and molecular mechanisms involved in the inflammatory process has increased in recent decades, and this has permitted the discovery of many promising targets for the development of new bioactive compounds to treat chronic inflammatory diseases.[6]

Arctium lappa (AL), commonly known as burdock, has anti-inflammatory,[7] free radical scavenging,[8] antioxidant,[9] and liver[10] and skin[11] protective effects.

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On lipopolysaccharide (LPS)-stimulated macrophages, arctigenin exhibited potent inhibitory effects on the production of nitric oxide (NO) and the release of tumor necrosis factor α (TNFα) and interleukin 6 (IL6), but not cyclooxygenase 2 (COX2) expression or COX2 activity.[12]

Camellia sinensis (CS), as green tea, is a species of plant whose leaves and leaf buds are used to produce the popular tea beverage. It is widely known for its anti-inflammatory effect, and there is growing interest in its cardiovascular health benefits.[13] Green tea and its major ingredient, epigallocatechin gallate, effectively inhibited the release of high mobility group box 1 (HMGB1) in LPS-induced macrophage cultures[14] by promoting its aggregation and autophagic degradation in these cells.[15]

Echinacea spp. plant preparations are claimed to have several pharmacological effects, such as immune enhancement, anti-inflammation, and prevention of common cold.[16] Numerous reports have demonstrated that *Echinacea* spp. constituents have inflammation-related bioactivities such as stimulation of cytokine production[17] or inhibition of COX1 or COX2 activity in vitro.[18] Hou et al,[19] in murine macrophages, demonstrated that alkamides significantly inhibited COX2 activity and the LPS-induced expression of COX2, inducible NO synthase (NOS2), and specific cytokines or chemokines.

Eleutherococcus senticosus (ES) is used as an immunological modulator in Oriental medicine.[20] It has been used to treat stress-induced physiological changes and various allergic disorders.[21–23] However, it is still unclear how it inhibits allergic responses and how effective it is in experimental models. On activated murine macrophage RAW264.7 cells, ES downregulated *NOS2* expression, showing pro-inflammatory action by blocking activation of JNK and Akt signaling pathways.[20]

The beneficial effects of *Panax ginseng* (PG), have been attributed to the biological activities of its constituents, the ginsenosides. Ginseng extracts and ginsenosides have been reported to have anti-inflammatory properties.[24] Ginseng can modulate the generation of inflammatory mediators (such as NO, synthesized by the activity of NOSs[25]) and the phagocytic activity of macrophages.[26]

Vaccinium myrtillus (VM) was one of the most frequently used antidiabetic remedies before the discovery of insulin.[27] However, Hou et al[28] demonstrated that delphinidin, one of the five anthocyanidins present in bilberry, suppressed COX2 by blocking MAPK-mediated pathways with the attendant activation of nuclear factor κB (NFκB), activator protein 1 (AP1), and CCAAT-enhancer-binding protein delta (C/EBPδ) in LPS-activated murine macrophage RAW264.7 cells. Moreover, gene expression profiling of inflammatory cell model RAW264.7 treated with bilberry extract was provided, showing that bilberry extracts affected genes related to inflammation and defense.[29]

A broad range of roles of secondary plant metabolites has been identified for several inflammatory cells and for a large number of inflammatory mediators in important pathologies not previously known to be linked to inflammation, such as obesity, atherosclerosis, and cancer.[30,31] However, evidence exists that nutraceuticals can modulate various inflammatory mediators (eg, cytokines), the production and/or action of second messengers (cGMP, cAMP, protein kinases), the expression of transcription factors (AP1, NFκB), and the expression of key proinflammatory molecules such as NOS2, COX2, interleukin 1β (IL1β) and TNFα, neuropeptides (substance P), and proteases (matrix proteases).[6] Moreover, whereas the NFκB plays an important role as transcription factor in coordinating the expression of proinflammatory genes, the peroxisome proliferator-activated receptor γ (PPARγ) plays an important role in controlling various diseases based on its anti-inflammatory properties.[32,33] In this regard, expression of PPARγ is not restricted to adipocytes, but is also found in immune cells, such as B and T lymphocytes, monocytes, macrophages, dendritic cells, and granulocytes.[34] All this evidence suggests a role of PPARγ in the control of the inflammatory response with potential therapeutic applications in inflammation-related diseases.[35]

It has been shown that nuclear factor E2-related factor 2 (NFE2L2) is responsible for both constitutive and inducible expression of antioxidant response element-regulated genes,[36] indicating an important protective role in regulating antioxidant and anti-inflammatory cellular responses.

In this regard, the aim of this research was to investigate the molecular activity of AL, CS, *Echinacea angustifolia* (EA), ES, PG, and VM extracts on cell model RAW264.7, in order to investigate their effect in modulating messenger (m)RNA expression of genes involved in the inflammatory process: *TNFα, COX2, IL1β, NFκB1, NFκB2, NOS2, NFE2L2*, and *PPARγ*. RAW264.7 cells were pre-stimulated with H_2O_2 to mimic inflammatory conditions and then treated with plant extracts.

Materials and methods

Plant extracts

All the extracts were obtained from commercial companies, standardized for the content of bioactive compounds (Table 1). AL was provided by Bayer S.p.A. (Milan, Italy), and CS, EA, ES, PG, and VM were provided by ACEF S.p.a. (Piacenza, Italy). The extracts (50 mg) were dissolved in 1 mL of 10% dimethyl sulfoxide (DMSO), passed through 0.22 μm pore-sized membrane filter (EMD Millipore, Billerica, MA, USA), and kept in the dark at −20°C until further analysis.

Table 1 Abbreviation, botanical name, used part, and yield of extract of each screened plant

Extract abbreviations	Botanical name	Used part	Origin	Content (HPLC method)	Extract yield (volume/volume)	
					Water	Ethanol
AL	*Arctium lappa*	Root	Italy	40% arctigenin	100	–
CS	*Camellia sinensis*	Leaf	Asia	≥95% polyphenols, ≥70% catechins total (≥40% EGCG)	–	100
EA	*Echinacea angustifolia*	Root	Italy	≥4% echinacoside	20	80
ES	*Eleutherococcus senticosus*	Root	Asia	2% eleutherosides	50	50
PG	*Panax ginseng*	Root	People's Republic of China	≥30% ginsenosides tot	50	50
VM	*Vaccinium myrtillus*	Fruit	Italy	≥25% anthocyanins	40	60

Abbreviations: EGCG, epigallocatechin gallate; HPLC, high-performance liquid chromatography.

Cell culture

RAW264.7 murine macrophages (American Type Culture Collection [ATCC], Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. All reagents were purchased from Euroclone (Milan, Italy). Cells were maintained in humidified air with 5% CO_2 at 37°C. For all experiments, the cells were grown to approximately 70%–90% confluence and culture medium with 2% heat-inactivated fetal bovine serum was used.[37]

H_2O_2 cell treatment

A preliminary assay was performed by treating macrophages with different final concentrations of H_2O_2 (50, 100, and 200 µM) diluted in phosphate-buffered saline (PBS) and further diluted in culture medium. The control cells (CTRL) were incubated for 24 hours with the same final amount of <0.1% DMSO in the culture medium.

This assay was used to identify the concentration of H_2O_2 that would be used to induce an inflammatory condition in the subsequent experiments. Cell viability was measured after 30 and 60 minutes of incubation with H_2O_2 (50, 100, and 200 µM).

Plant extracts cell treatment

To test the effect of plant extracts on macrophages, cells were washed with PBS and treated with different doses of plant extract (1, 10, 100, and 200 µg/mL) in a final concentration of <0.1% DMSO in the culture medium for 24 hours. In addition, a combined effect of each plant extract and H_2O_2 was assayed by incubating cells for 60 minutes with H_2O_2 (200 µM) and, after washing with PBS, with the specific dose of each plant extract in a final concentration of <0.1% DMSO in the culture medium for 24 hours. The CTRL were incubated for 24 hours with the same final amount of <0.1% DMSO in the culture medium.

Cell viability assay

To ascertain the cytotoxic effect of H_2O_2 and plant extracts, viability of RAW264.7 cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay.[11,38] For the assay, cells were seeded in a 96-well plate at a density of 2×10^4 cells/well and left to grow for 24 hours. Per well, 10 µL of MTT reagent was added and the mixture was further incubated for 3 hours. Next, the mixture in each well was removed, and formed formazan crystals were dissolved in 100 µL of DMSO. Optical density measurement of the mixture was performed in a microplate reader (Tecan GENios, Männedorf, Switzerland) at 550 nm. Cell viability was expressed as percentage of each treatment versus the CTRL.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

To determine the modulation of H_2O_2 on mRNA expression of *IL1β, NFE2L2, NFκB1, NFκB2, PPARγ, NOS2, COX2*, and *TNFα*, qRT-PCR was preliminarily performed on macrophages treated with 50, 100, and 200 µM H_2O_2 for 30 and 60 minutes.

In addition, macrophages were treated with 10 µg/mL of each extract for 24 hours after a pre-stimulation using 200 µM H_2O_2 for 60 minutes. To determine whether plant extracts had an effect on H_2O_2 pre-stimulated cells, the mRNA expression of the same genes was analyzed.

After treatment, cells were washed with cold PBS before total RNA extraction using the RNeasy Plus Mini Kit (Qiagen NV, Venlo, the Netherlands), according to the manufacturer's instructions. Then, complementary (c)DNA was synthesized from the same purified RNA samples according to ImProm-II™ Reverse Transcription System (Promega Corporation, Fitchburg, WI, USA) and using random and oligo$(dT)_{15}$ primers. Briefly, 1 µg of the purified total RNA was denatured by incubating at 70°C for 5 minutes and then placed at 25°C for 5 minutes, 42°C for 60 minutes, and 70°C for 15 minutes.

Primer3 Input software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA)[39] was used to design the primer sequences encoding for *β actin* (*ACTB*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *IL1β, NFE2L2, NFkB1, NFkB2, PPARγ, NOS2, COX2*, and *TNFα* (Table 2). The relative qRT-PCR was performed in triplicate with the Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Life Technologies, Carlsbad, CA, USA) on CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Reaction mixes were prepared with 200 nM of each primer pair (Table 2) and the cDNA (10 ng) added last. Differences between samples and controls were calculated using the $2^{-\Delta\Delta Ct}$ method,[40,41] where $2^{-\Delta\Delta Ct}$ represents the difference of a given target gene in H_2O_2-treated cells versus the CTRL (Table 3) and each sample treated with plant extract versus H_2O_2-treated cells (Figure 1). The expression of target genes was normalized using *ACTB* and *GAPDH*, and ΔCts were calculated by the difference between Ct of the gene target and the geometric mean of the two housekeeping genes. The n-fold expression of a given target gene was calculated as $\log_2(2^{-\Delta\Delta Ct})$.

Statistical analysis

Cell viability data related to H_2O_2 treatments and computed as % mean ± standard error (SE), were analyzed using the analysis of variance (ANOVA) model to assess the significance of doses (50, 100, and 200 μM H_2O_2) and time of treatments (30 minutes and 60 minutes). The effect of plant extracts on cell viability was analyzed using the ANOVA model. The differences in cell viability were analyzed among doses (1, 10, 100, and 200 μg/mL) of each extract. Means were compared adjusting the *P*-values with Bonferroni correction for multiple comparisons (Statistical Package for the Social Sciences [SPSS] Version 16.0; SPSS Inc., Chicago, USA).

Relative gene expression data, after H_2O_2 treatments, were analyzed using the general linear model to assess the significance of doses (50, 100, and 200 μM H_2O_2) and time of treatments (30 minutes and 60 minutes). Expression data are reported as a mean of $\log_2$(n-fold) ± SE. Means were compared adjusting *P*-values with Bonferroni correction for multiple comparisons (SPSS Version 16.0; SPSS Inc.).

The effect of plant extracts on gene expressions, computed as $\log_2$(n-fold), were checked by the general linear model. The differences within each gene were analyzed among different extracts. Data were reported as mean of $\log_2$(n-fold) ± SE. For mean comparisons, the *P*-values were adjusted with Bonferroni correction for multiple comparisons (SPSS Version 16.0; SPSS Inc.).

Results

Cell viability

To analyze the effect of dose and time of H_2O_2 exposure, a preliminary cell viability assay was performed by treating macrophages with increasing doses of H_2O_2 from 1 to 200 μM at two times of incubation (30 minutes and 60 minutes). Cell viability

Table 2 Primer pair sets and parameters used in the real time polymerase chain reaction analysis

Gene	GenBank accession number	Sequence (5′ → 3′)	Product length (bp)	Primer (nM)	Tan (°C)
COX2	AK134081	For: CCCACAGTCAAAGACACTCA Rev: CTCATCACCCCACTCAGGA	189	200	57
IL1β	AK225002	For: GACCTTCCAGGATGAGGACA Rev: AGCTCATATGGGTCCGACAG	183	200	58
NFE2L2	BC152371	For: GGTTATCACAGCTGCCTG Rev: GGTAGTTCTGGGACATGG	253	200	56
NFκB1	AK090099	For: CTGACCTGAGCCTTCTGGAC Rev: GCAGGCTATTGCTCATCACA	177	200	59
NFκB2	AK144994	For: TGACTGTGGAGCTGAAGTGG Rev: GGTGTGTTTCCAGCAAAGGT	153	200	58
NOS2	M87039	For: CACCTTGGAGTTCACCCAGT Rev: ACCACTCGTACTTGGGATGC	170	200	59
PPARγ	U01841	For: GAGCTGACCCAATGGTTG Rev: ACCCTTGCATCCTTCACAAG	232	200	57
TNFα	AK155964	For: CGAGTGACAAGCCTGTAGCC Rev: GTGGGTGAGGAGCACGTAGT	209	200	60
ACTB	NM_007393	For: CACCAACTGGGACGACATGGAG Rev: GGCCTGGATGGCCACGTACAT	203	200	59
GAPDH	NM_008084	For: AATGTGTCCGTCGTGGATCTGA Rev: AGTGTAGCCCAAGATGCCCTTC	117	200	60

Abbreviations: For, forward; Rev, reverse; Tan, annealing temperature; *COX2, cyclooxygenase 2; IL1β, interleukin 1β; NFE2L2, nuclear factor E2-related factor 2; NFκB, nuclear factor κB; NOS2, nitric oxide synthase 2; PPARγ, proliferator-activated receptor γ; TNFα, tumor necrosis factor α; ACTB, β actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.*

Table 3 messengerRNA expression of *TNFα*, *IL1β*, *NFκB1*, *NFκB2*, *NOS2*, *COX2*, *NFE2L2*, and *PPARγ* in RAW264.7 cells treated with 50, 100, and 200 μM of H_2O_2 for 30 and 60 minutes in comparison to control cells

Gene	Dose (μM)	30 minutes	60 minutes	Mean squared error	Time	Dose	Time × dose
		log_2(n-fold)					
TNFα	50	0.88	0.98	0.225	0.012	0.009	0.207
	100	1.07	1.81				
	200	1.39	2.52				
IL1β	50	1.23	0.86	0.405	0.817	0.746	0.551
	100	1.21	1.36				
	200	1.08	1.52				
NFκB1	50	0.89	1.56	0.464	0.000	0.008	0.124
	100	1.05	3.03				
	200	1.57	3.89				
NFκB2	50	0.97	1.74	0.114	0.000	0.002	0.044
	100	1.17	2.33				
	200	1.36	3.23				
NOS2	50	0.91	1.15	0.342	0.35	0.266	0.025
	100	1.69	1.53				
	200	0.43	2.3				
COX2	50	0.55	1.69	0.096	0.000	0.000	0.000
	100	0.21	2.6				
	200	0.79	4.05				
NFE2L2	50	0.13	2.73	0.206	0.000	0.002	0.013
	100	2.03	3.16				
	200	0.85	3.70				
PPARγ	50	−0.56	−0.61	0.108	0.694	0.150	0.956
	100	−0.72	−0.73				
	200	−0.92	−1.04				

Notes: Data are presented as the log_2(n-fold) mean ± standard error (n=3). The significance of time of incubation (30 and 60 minutes) and doses, and of the interaction between time × dose (determined by the analysis of variance), are reported.

Abbreviations: *COX2, cyclooxygenase 2; IL1β, interleukin 1β; NFE2L2, nuclear factor E2-related factor 2; NFκB, nuclear factor κB; NOS2, nitric oxide synthase 2; PPARγ, proliferator-activated receptor γ; TNFα, tumor necrosis factor α.*

(% versus CTRL) after exposure for 30 minutes and 60 minutes slowly decreased, remaining at over 60% ($P<0.001$) up to 200 μM (67.89%±1.66%) (Table 4). The dose of 200 μM of H_2O_2 was chosen to evoke inflammation for 60 minutes without affecting the cell viability (see also the next paragraph).

In addition, cells were treated for 24 hours with plant extracts (Table 5). A concentration of 10 μg/mL of each AL, CS, EA, ES, PG, and VM significantly ($P<0.001$) reduced cell viability in comparison to the CTRL, remaining, nevertheless, over 80%. On the contrary, concentrations of 100 and 200 μg/mL led to a strong decrease ($P<0.001$) in viability. Based on this evidence, 10 μg/mL of each plant extract was chosen for the further experiments.

H_2O_2 upregulates mRNA expression of inflammatory genes in macrophages

As shown in Table 3, the H_2O_2 treatment induced the upregulation of all genes, except *PPARγ*, at both incubation times. In particular, the mRNA expression of *NFkB1, NFkB2, COX2*, and *NFE2L2* significantly increased ($P<0.001$) after 60 minutes of incubation. *TNFα* expression was significantly induced at $P<0.05$. The mRNA expression of *COX2* was also significantly ($P<0.001$) upregulated in a concentration-dependent manner. The mRNA expression of *TNFα, NFkB1, NFkB2*, and *NFE2L2* showed a similar trend, at $P<0.01$. The interaction between time and dose of incubation was significant in the expression of *COX2* ($P<0.001$) and *NFkB2, NOS2*, and *NFE2L2* ($P<0.05$). The relative expression of *IL1β* and *PPARγ* did not present any significant variation between doses, times of incubation, or in the interaction between time and dose. Taken together, these results show that 60 minutes of exposure and a concentration of 200 μM were significantly more effective than the other times or concentrations in stimulating the mRNA expression, and therefore were chosen for the further experiments.

Plant extracts modulate the expression of cytokines in pretreated H_2O_2 macrophages

As shown in Figure 1, all of the treatments significantly ($P<0.001$) modulated the mRNA expression of each gene, but with some specific effect. AL showed an increase of *NOS2, NFE2L2*, and *PPARγ* expression, while depressing the expression of *TNFα, IL1β, COX2, NFkB1*, and *NFkB2*. CS stimulated *COX2, NFE2L2*, and

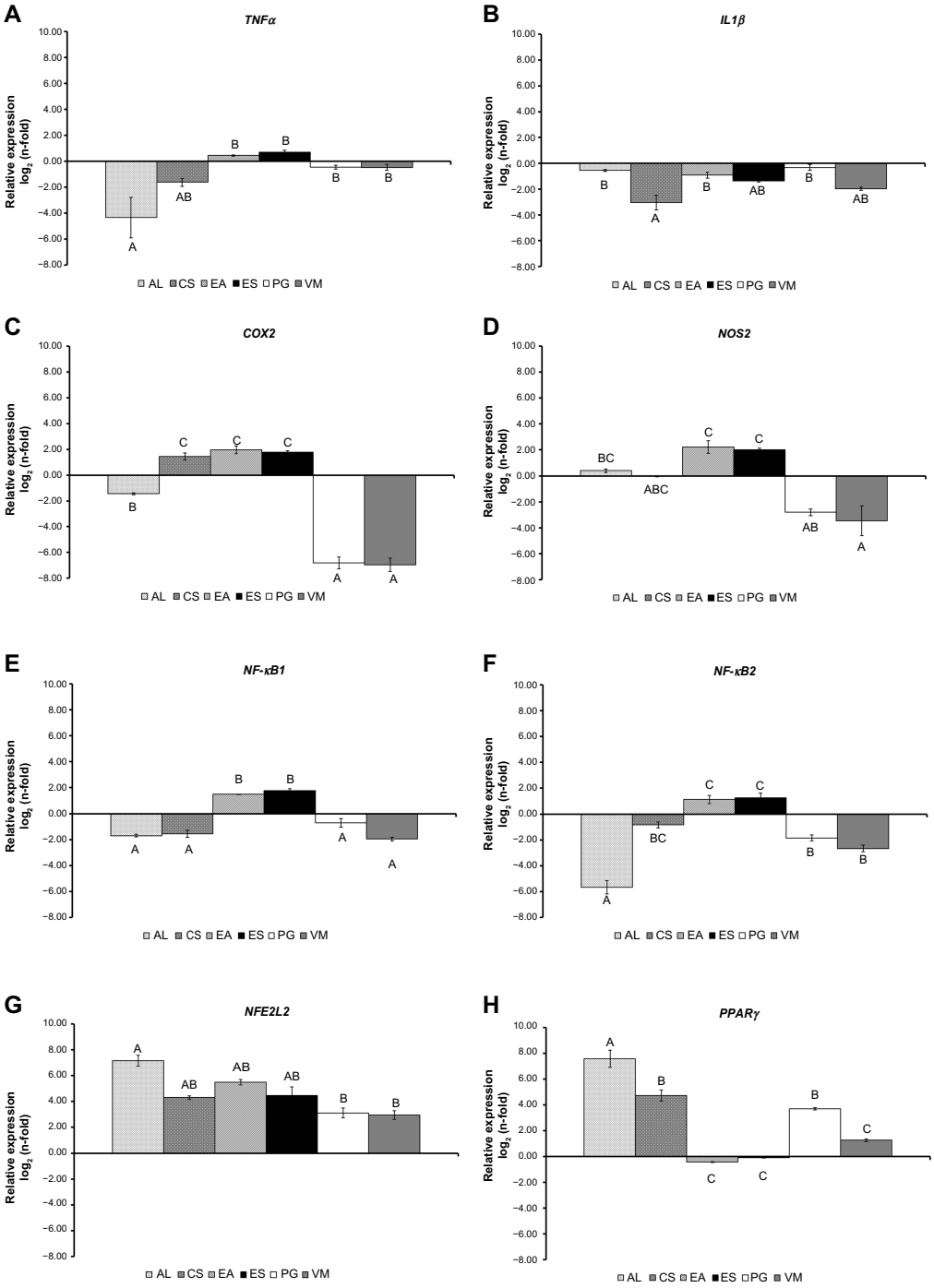


Figure 1 messengerRNA expression of *TNFα*, *IL1β*, *COX2*, *NOS2*, *NFκB1*, *NFκB2*, *NFE2L2*, and *PPARγ* in RAW264.7 cells treated with 10 μg/mL of each extract for 24 hours after a pre-stimulation using 200 μM H_2O_2 for 1 hour relative to H_2O_2-treated cells.

Notes: (**A**) *TNFα*; (**B**) *IL1β*; (**C**) *COX2*; (**D**) *NOS2*; (**E**) *NFκB1*; (**F**) *NFκB2*; (**G**) *NFE2L2*; (**H**) *PPARγ*. To compare the bioactivities between six plant extracts for each gene, the general linear model was used with fixed effect for extracts. Bars represent the $\log_2$(n-fold) mean ± standard error (n=4). The effect of plant extracts for each gene are indicated with different capital letters denoting significant differences at $P<0.001$.

Abbreviations: AL, *Arctium lappa*; CS, *Camellia sinensis*; EA, *Echinacea angustifolia*; ES, *Eleutherococcus senticosus*; PG, *Panax ginseng*; VM, *Vaccinium myrtillus*; *COX2, cyclooxygenase 2; IL1β, interleukin 1β; NFE2L2, nuclear factor E2-related factor 2; NFκB, nuclear factor κB; NOS2, nitric oxide synthase 2; PPARγ, proliferator-activated receptor γ; TNFα, tumor necrosis factor α.*

PPARγ, and negatively modulated *TNFα, IL1β, NOS2, NFκB1*, and *NFκB2*. EA and ES upregulated all genes except *IL1β* and *PPARγ*. PG and VM caused a decreased expression of all genes apart from *NFE2L2* and *PPARγ*, which were stimulated.

Discussion

Epidemiological studies have provided convincing evidence that natural dietary compounds possess many biological activities against various chronic inflammatory diseases.[42,43] In the present study, we evaluated the effect of AL, CS, EA, ES, PG,

Table 4 Cell viability of RAW264.7 cells treated with 50, 100, and 200 μg/mL of H_2O_2 for 30 and 60 minutes

Concentration (μg/mL)	30 minutes	60 minutes
	Mean ± SE	Mean ± SE
0	100.00^{A}±0.00	100.00^{A}±0.00
50	$86.24^{A,B}$±1.42	$84.38^{A,B}$±1.75
100	78.06^{B}±1.17	73.23^{B}±3.15
200	76.03^{B}±0.23	67.89^{B}±1.66

Notes: Data are presented as % mean ± standard error (n=6). Different superscript letters indicate significant ($P<0.001$) effects of doses within times of incubation.
Abbreviation: SE, standard error.

and VM extracts on the expression of relevant genes involved in inflammatory response. The analyses were performed on macrophages, key cells of immunity mechanisms.[44] In particular, these cells are essential in the shift of the immune response from the so-called type 1 response with cytotoxic effector cells toward the type 2 response that is characterized by antibody production. Previous studies have been commonly conducted on LPS-induced macrophages, while, in this paper, macrophages were stimulated by direct addition of H_2O_2. In fact, H_2O_2 acts as a secondary messenger in diverse stimuli, which are also not infective, that activate NFκB.[45] In this study, the data demonstrated that 200 μM of H_2O_2 affects cell viability similarly to lower doses. Moreover, the choice of 200 μM H_2O_2 is a good compromise between the doses that activate inflammatory mediators, such as NFκB, and those naturally produced in the inflammatory process.[46] In fact, the addition of 200 μM H_2O_2 for 60 minutes on RAW264.7 cells significantly stimulated the transcription of *TNFα, COX2, NFkB1, NFkB2, NOS2*, and *NFE2L2*. Interestingly, no differences were found between times of exposure and doses in *IL1β* and *PPARγ* expression. H_2O_2 clearly stimulated the expression of NFκB-dependent genes in RAW264.7 cells, likely induced by proinflammatory cytokine TNFα, as already observed in MCF-7 cells.[47,48] Moreover, it is known that several proteins can be oxidized and thus modified by H_2O_2. These redox-regulated proteins include transcription factors such as p53, Jun, Fos, and the p50 subunit of NFκB (NFκB1). The oxidation of these proteins can either prevent (*p53, Jun, Fos*) or stimulate (*p50*) the transcriptional activity of these genes.[49]

Table 5 Cell viability of RAW264.7 cells treated with 1, 10, 100, and 200 μg/mL plant extracts for 24 hours

Extract	Concentration (μg/mL)			
	1	10	100	200
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
AL	98.82^{A}±1.64	97.78^{A}±1.73	56.34^{B}±0.86	44.46^{C}±1.16
CS	95.97^{A}±0.00	89.14^{A}±0.00	56.95^{B}±0.00	32.97^{C}±0.00
EA	95.98^{A}±1.50	93.88^{A}±1.22	74.14^{B}±3.19	39.36^{C}±1.63
ES	89.31^{A}±0.93	86.59^{A}±1.44	55.81^{B}±1.39	34.66^{C}±1.44
PG	94.21^{A}±1.44	87.48^{A}±1.52	55.48^{B}±1.80	54.44^{B}±0.90
VM	99.00^{A}±1.12	95.53^{A}±2.33	77.95^{B}±1.08	49.12^{C}±1.08

Notes: Data are presented as % mean ± standard error (n=6). Different superscript letters indicate significant differences ($P<0.001$) within treatments at different concentrations (within each row).
Abbreviations: AL, *Arctium lappa*; CS, *Camellia sinensis*; EA, *Echinacea angustifolia*; ES, *Eleutherococcus senticosus*; PG, *Panax ginseng*; SE, standard error; VM, *Vaccinium myrtillus*.

The plant extract treatments on pre-stimulated macrophages showed an anti-inflammatory action, downregulating inflammatory genes that typically can activate NFκB1 and NFκB2 pathways, as previously observed.[12,50]

Flavonoids, which specifically affect the modulation of inflammatory gene expression and the function of enzymes critically involved in inflammation,[51,52] are also present in AL,[53] CS,[54] PG,[55] and VM.[56]

Zhao et al,[12] using LPS to stimulate RAW264.7 cells, demonstrated a block in NO production after addition of the active compound arctigenin. Moreover, the major polyphenol in CS extracts, epigallocatechin gallate, has been recently found to suppress *NOS2* mRNA and protein expression in macrophages[57] through the inhibition of the activation of *NFκB*.[58] In the present study, *NOS2* was upregulated by AL, and by up to twofold by EA and ES, in comparison to the control. CS downregulated *NOS2* expression related to the control, possibly through inhibition of *NFκB1* and *NFκB2*, but without any significant difference compared to other compounds. In general, controversial data could be due to the different extraction solvent, stimulant (LPS or H_2O_2), and different active compounds present in the extract.

Anthocyanins, from bilberry (VM), constitute the major bioactive components responsible for the observed downregulating effects on genes involved in inflammatory response, as already observed in a microarray study.[29] Among the tested extracts, VM showed the highest anti-inflammatory activity, as also reported in LPS-stimulated human monocytic cell line by downregulating all the proinflammatory genes, in particular *COX2* and *NOS2*.[59] It has previously been shown that, in murine RAW264.7 cells, anthocyanidins inhibited LPS-induced *COX2* expression by blocking MAPK pathways with the attendant activation of NFκB, C/EBPδ, and AP1.[28]

EA and ES seem to act as immunostimulating agents, downregulating *IL1β* only. It is known that *Echinacea spp*. plant extract has been used for immunomodulation,[60] but the evidence supporting its therapeutic potential is still controversial. In vitro studies have shown that the commercial preparations of *Echinacea spp*. extract stimulate cytokine production by human peripheral blood macrophages[17] and enhance NK cell function and antibody-dependent cytotoxicity of peripheral blood mononuclear cells.[61,62] In

the present study, EA caused an upregulation of *NFkBs, TNFα*, and *COX2*. An enhanced release of cytokines (such as TNFα and interferon-γ) in response to *Echinacea spp*. components was also detectable in rat spleen macrophage.[60] The existing results suggest that the *Echinacea spp*. preparations are potentially effective in stimulating an in vitro and in vivo nonspecific immune response.[63,64]

Similarly to EA, ES downregulated *IL1β* only. Previous reports indicated that the isolated compounds from ES inhibit the NOS2 as well as COX2 expression.[65,66] However, it has also been shown that 80% ethanol extract from ES attenuated LPS-induced NOS2 expression but not COX2 expression.[20] Dissimilar results may be due to efficacy according to concentration between whole extract and its derived compounds. On the other hand, EA and ES are the only extracts that downregulated the expression of *PPARγ*, therefore promoting the expression of inflammatory mediators. The PPARγ, a member of the nuclear hormone receptor superfamily, is essential for adipocyte differentiation and glucose homeostasis. PPARγ has been found to regulate macrophage activation in response to mitogens and inflammation,[67] and it was recognized as a negative regulator of the inflammatory activation of both macrophage and T-cells.[68] Christensen et al[69] demonstrated that, on 3T3-L1 adipocytes, various fatty acids and alkylamides from the flowers of *Echinacea purpurea* at high concentrations activated PPARγ. An involvement of PPARγ in the inhibition of IL2 secretion by T-cells in response to EA alkylamides has also been observed.[70] Interestingly, only EA and ES did not stimulate the expression of *PPARγ*, and this was in agreement with the attendant overexpression of the proinflammatory genes.

EA and ES, as well as AL, CS, PG, and VM, upregulated *NFE2L2*, a transcriptional regulator of antioxidant enzymes with an important role in attenuating oxidative stress associated with diseases.[71] CS has been shown to activate NFE2L2 in HL-1 murine cardiomyocytes and to induce phase II enzymes through the antioxidant response element and concomitant protection against H_2O_2.[72] Furthermore, echinacoside, one of the major active compounds in EA,[73] resulted in strong activation of NFE2L2 in HaCaT cells, providing a potential skin protection.[74] Interestingly, in the present study, phytochemical treatments modulated the *NFE2L2* expression, which was increased by the oxidant treatment.

Conclusion

Although the important role that H_2O_2 plays as an intracellular messenger is not well understood, it is known that local H_2O_2 concentration in extracellular fluids during inflammatory processes are sufficient to activate the NFκB pathway, thereby allowing induction of the synthesis of cytokines and other inflammatory mediators. The stimulation of H_2O_2 on RAW264.7 cells induced the transcription of proinflammatory mediators, showing that this could be an applicable system by which to activate macrophages. The extracts from AL, CS, PG, and VM decreased the expression of proinflammatory genes on H_2O_2-induced RAW264.7 cells in vitro, while EA and ES, on the contrary, promoted these inflammatory mediators.

The promising anti-inflammatory molecular mechanisms of plant extracts in macrophages of murine RAW264.7 cells require validation in future in vivo dietary intervention studies.

Acknowledgments

This work was supported by Progetto Nutriheart POR FESR 2007–2013 Friuli Venezia Giulia, Italy.

Author contributions

Elena Pomari performed the experiments concerning cell viability, isolation of RNA, and qRT-PCR. Moreover, she assisted with interpretation of the results and helped draft the manuscript. Bruno Stefanon and Monica Colitti assisted with interpretation of the results, supervised the analyses, and helped to draft the manuscript. All authors read and approved the final manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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