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Analysis of the anti-inflammatory potential of *Brassica* bioactive compounds in a human macrophage-like cell model derived from HL-60 cells

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ABSTRACT

Background: Chronic inflammatory diseases are major causes of global morbidity and mortality. Acute inflammation is meant to protect the body against foreign agents, but it also plays a major role in tissue repairment. Several mediators are involved in this process, including pro-inflammatory cytokines produced by macrophages. Occasionally, if the inflammatory response is not resolved, the acute inflammatory process can evolve into a chronic inflammation. Natural compounds from vegetables are considered as an important source of active agents with potential to treat or prevent inflammatory related pathologies and could be used as an alternative of the therapeutic agents currently in use, such as non-steroidal anti-inflammatory drugs (NSAIDs), which present several side effects.

Methods: In this research work we evaluated *in vitro* the anti-inflammatory activity of a series of ten phytochemicals present in *Brassica*, measured as the potential of those compounds to reduce the production of key pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) by a human macrophage-like cell model of HL-60 cells

Results: Most of the tested phytochemicals (including the most representative bioactive molecules of the major classes of compounds present in cruciferous foods such as glucosinolates, isothiocyanates, hydroxycinnamic acids, flavonols and anthocyanins) demonstrated significant anti-inflammatory activity at micromolar level in the absence of cytotoxic effects in this human macrophage-like cell model.

Conclusion: These data confirm that phytochemicals commonly obtained from *Brassica* may be potential therapeutic leads to treat or prevent human chronic inflammation and related diseases.

1. Introduction

Chronic inflammatory diseases have become major causes of global morbidity and mortality [1]. Inflammation is a natural process coordinated by the immune system, which primary function is to maintain the organism homeostasis. The inflammatory process is meant to protect the

Abbreviations: CQA, Chlorogenic acid (5-Caffeoyl-quinic acid); Cy-3glc, Cyanidin-3-glucoside; CCM, cell-culture medium; DIM, 3,3′-Diindoylmethane; GBS, Glucobrassicin; GRE, Glucoraphenin; GRA, Glucoraphanin; GER, Glucoerucin; GSLs, glucosinolates; I3C, Indole-3-Carbinol; IL-1β, interleukin 1 beta; IL-6, interleukin 6; ITCs, isothiocyanates; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, nuclear factor erythroid 2–related factor 2; NSAIDs, non-steroidal anti-inflammatory drugs; SIA;, Sinapic acid (Sinapinic acid); SFN, sulforaphane; TNF-α, tumor necrosis factor alpha.

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body against the attack of foreign agents, but it is also activated in response to other endogenous signals, such as tissue damage components produced by injuries, thus playing a major role in tissue repairment [2]. A wide series of molecular mediators are involved in this process, including proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL) 6 (IL-6) and interleukin 1 beta (IL-1β), which are produced by macrophages [3,4]. Occasionally, if the inflammatory response is not correctly resolved, the acute inflammatory process can evolve into a chronic inflammation, which in turn can lead to different related diseases such as rheumatoid arthritis, type 2 diabetes, cardiovascular and neurodegenerative diseases, and asthma, among others [5]. Furthermore, the existence of a direct relationship between the inflammatory process and cancer has also been established [6–8], making the need for the study of this process' pathogeny in humans even more relevant from both the clinical and biomedical points of view.

Among the currently available agents to treat inflammatory diseases, non-steroidal anti-inflammatory drugs (NSAIDs) have been for years the main therapeutic option due to their ability to block cyclooxygenases involved in the synthesis of important inflammatory mediators such as prostaglandins [9]. However, the development of alternative strategies to NSAIDs is still crucial due to the important adverse effects that these drugs present, which include mainly gastrointestinal, cardiovascular, and renal toxicity [10,11]. Thus, in this scenario, the search for novel therapeutic agents to prevent or treat these inflammatory related pathologies has become a major research target. Nevertheless, although a great effort has been done in this field of research, not many potentially active molecules have reached pharmacological or clinical use to date [12], and so there is still a need for the search of novel therapeutic agents to treat human inflammatory diseases with none or minor collateral effects.

Remarkably, natural compounds from vegetables and their derivatives are considered as an important source of active agents with a great potential to treat or prevent these pathologies. Vegetables are not only nutritious, but also rich sources of potentially active phytochemicals. In particular, the *Brassicaceae* family, which includes well known and socioeconomically relevant crops such as broccoli, cabbages, radishes, mustard greens, *etc.* [13], are very rich in relevant biomolecules. Some of these potentially bioactive phytochemicals, such as the glucosinolates (GSLs), are in fact almost exclusively present in *Brassica* [14]. The GSLs consist on a family of compounds containing a basic structure of a thiohydrozimate-O-sulfonate group with a glycosylation and a different side-chain, depending on which amino acid they are derived from [15]. The GSLs are stable secondary metabolites, but after tissue disruption, they are hydrolyzed by the enzyme myrosinase (EC 3.2.1.147), generating isothiocyanates (ITCs), which have been shown to be the main bioactive molecules with diverse bioactivities [16–21]. It has been reported that (ITCs) are strong inducers of Phase II detoxification enzymes, which are partially responsible for their anti-inflammatory properties [22]. Between aliphatic GLSs, glucoraphanin (GRA) is one of the most studied because its resultant ITC, sulforaphane (SFN), has shown diverse properties on human health. It has been described that SFN exerts its main function through the activation of the nuclear factor erythroid 2–related factor 2 (Nrf2), as it binds to Nrf2 natural repressor Keap-1, allowing thus the transcription of Phase II protective enzymes [23]. Furthermore, SFN is also able to interfere with the activity of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), decreasing its capability to bind target sequences related to inflammatory processes, such as those codifying for TNF-α [20,24].

Aliphatic glucosinolates such as glucoraphanin (GRE) or glucoerucin (GER) have also been evaluated for interesting bioactivities in the energy metabolism, with positive metabolic effects in glucose [25] or obesity [26], respectively. Another abundant GSL present in *Brassica* vegetables is glucobrassicin (GBS) [27], which is the precursor of indole-3-carbinol (I3C). However, I3C is a relative unstable compound, so it usually dimerizes into the condensation product 3,3-diindolylmethane (DIM) [28]. Although several studies showed that DIM interferes with diverse signal transduction pathways implied in inflammatory processes, such as protein kinase B (PKB, also known as AKT kinase), phosphoinositide 3 kinase (PI3K), the NFκB pathway or the epidermal growth factor receptor/extracellular-regulated kinase (EGFR/ERK) pathway, the exact mode of interaction has not been yet elucidated [29].

Other relevant natural compounds with health promoting qualities that are widely present in plants, plant-derived foods, and other derived products, are phenolic compounds [30,31]. These compounds range from simple, low molecular-weight, single aromatic-ringed structures to large and complex tannins and derived polyphenols. The most widely spread and diverse group of phenolics in cruciferous vegetables (*Brassicaceae* family) are the flavonoids (mainly flavonols, but also anthocyanins) and the hydroxycinnamic acids, mainly chlorogenic and sinapic acid derivatives [32].

The main hypothesis of this study postulates that several phytochemicals representative of the major classes of compounds present in cruciferous foods (GSLs, ITCs, hydroxycinnamic acids, flavonols and anthocyanins), may display a series of biological activities that would made them suitable to be used as potential therapeutic agents to treat or prevent relevant human inflammatory related diseases. To confirm this hypothesis, we stablished two main aims. First, to prove the utility of a human macrophages-like cells-based model derived from the HL-60 cell line to test the biological activity and safety of natural phytochemicals *in vitro*. Secondly, to analyze the anti-inflammatory potential of a variety of phytochemicals present in *Brassica* administrated at low optimal concentrations, with no toxic side effects.

2. Material and methods

2.1. Compounds

High-purity standards of the GSLs glucoraphanin (GRA), glucoraphenin (GRE), glucoerucin (GER), and glucobrassicin (GBS), as well as the phenolic compounds chlorogenic acid (CQA), sinapic acid (or sinapinic acid, SIA), and the cyanidin-3-glucoside chloride (Cy3-glc) were obtained from Phytoplan Diehm & Neuberger GmbH (Heidelberg, Germany). The ITC sulforaphane (SFN), and the indoles Indol-3-Carbinol (I3C) and 3,3′-Diindolmethane (DIM) were obtained from LKT Laboratories, Inc. (Minnesota, USA) (Table 1).

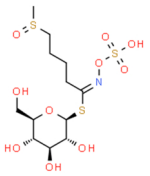
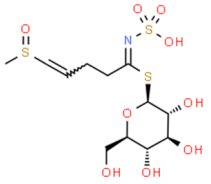
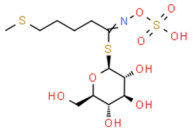
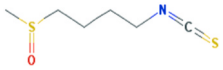
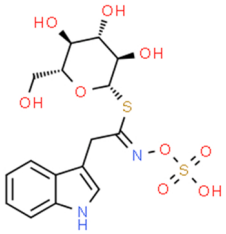
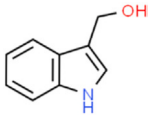
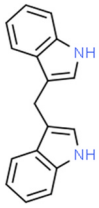
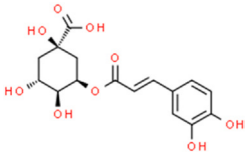
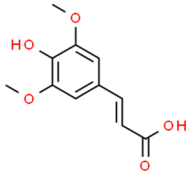
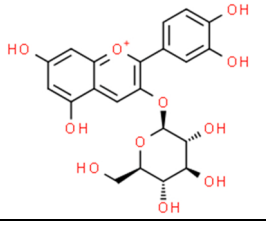
2.2. Preparation of compounds for in vitro assays

A stock solution at a concentration of 1 mg/mL for each of the tested compounds was freshly prepared in sterile-filter liquid phosphate buffered saline (PBS) pH= 7.4 (Biowest, Nuaillé, France), with the only exception of SFN, which was first diluted in dimethyl sulfoxide (DMSO) (Merk, Whitehouse Station, NJ, USA) to get a final stock solution of 1 mg/mL SFN in PBS containing 10% DMSO. Successive solutions were made at the appropriate concentration in complete cell-culture medium (CCM) prior to be used in the *in vitro* assays. Compounds were finally added to the cells to obtain the final assay concentrations.

2.3. Cell culture

Brassica derived phytochemicals were tested using the human acute myeloid leukemia cell line HL-60 (ATCC® CCL-240™) as the elected *in vitro* model. Cells were incubated in CCM consisting in RPMI-1640 (Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (GIBCO Invitrogen, Paisley, UK) and 1% penicillin/streptomycin (GIBCO), at 37 °C with 5% CO_2. All *in vitro* assays were performed after passage number 5 and before passage number 20 with cells growing at an exponential ratio.

Table 1
Brassica derived compounds.

Compound	Type	Abbreviation	Structure
Glucoraphanin	Aliphatic glucosinolate	GRA	
Glucoraphenin	Aliphatic glucosinolate	GRE	
Glucoerucin	Aliphatic glucosinolate	GER	
Sulforaphane	Isothiocyanate	SFN	
Glucobrassicin	Indolic glucosinolate	GBS	
Indole-3-Carbinol	Indole	I3C	
3,3′-Diindoylmethane	I3C metabolic dimer	DIM	
Chlorogenic acid (5-Caffeoyl-quinic acid)	Hydroxycinnamic acid	CQA	
Sinapic acid (Sinapinic acid)	Hydroxycinnamic acid	SIA	
Cyanidin-3-glucoside	Anthocyanin	Cy3glc	

2.4. In vitro anti-inflammatory assays

Anti-inflammatory activity of the phytochemicals was analyzed by determining their capacity to modulate *in vitro* the secretion of pro-inflammatory cytokines in inflammatory-like conditions induced by bacterial lipopolysaccharide (LPS). To do so, human myeloid leukemia HL-60 cells were differentiated to human macrophage-like cells by using a dose of 10 ng/mL phorbol myristate acetate (PMA) (Sigma Chemical Co., St. Louis, MO, USA) for a period of 24 h upon a ratio of $0.2{\cdot}10^6$ cells/well in 96-wells plates in CCM [33]. Cells then rested in CCM without PMA for another 24 h. After differentiation and resting periods, tested compounds (or vehicle) were added to cells at doses of 10 μM, 25 μM, and 50 μM, and plates were pre-incubated for 30 min at 37 °C. Finally, LPS (*Escherichia coli* 0111. B4; Sigma Chemical Co.) was added at a final concentration of 0.1 μg/mL to produce a low degree of pro-inflammatory conditions like those found in chronic inflammatory diseases. Plates were then incubated at 37 °C with 5% CO_2 for 24 h and supernatants were collected and kept at − 20 ºC until further analysis (Fig. 1).

2.5. Enzyme-linked immunosorbent assays

Supernatants from *in vitro* assays of macrophage-like cells were assayed to determine the levels of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β using enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's instructions (eBiosciences, San Diego, CA, USA). The absorbance in each well was measured with a microplate reader (Spectrostar Nano; BMG Labtech, Ortenberg, Germany) at 450 nm and corrected at 570 nm. Concentrations of cytokines secreted by the differentiated macrophage-like HL-60 cells were obtained by using the corresponding standard curve, and the modulation of cytokines production was measured and calculated as level of release inhibition compared to control conditions. To directly compare the phytochemicals' effects obtained from different experiments ($N \geq 2$), cytokines concentrations obtained by ELISA following different treatments (ranging from: 30–350 pg/mL for TNF-α; 250–800 pg/mL for IL-6; 30–120 pg/mL for IL-1β) were normalized for each singular experiment by using the negative control of the corresponding assay as referential point (Valued as 1 for control conditions). Finally, the effects upon cytokines' levels were displayed as the mean data ± SEM of normalized results. Since SFN has already demonstrated to be an anti-inflammatory agent in other *in vitro* models of inflammation [16,22,34], it was used as internal positive control once we confirmed its anti-inflammatory activity in the present cell model.

2.6. In vitro viability assays

Viability of differentiated cells in the presence of the compounds, measured as cells' metabolic capacity, was evaluated by MTT assays (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole, to purple formazan by cellular mitochondrial enzymes) [33]. In brief, after differentiation of human HL-60 to macrophage-like cells, these were treated with different doses of the tested compounds (10 μM, 25 μM, and 50 μM) plus LPS (pro-inflammatory treatment) for 24 h, MTT (Alfa Aesar, Thermo Fisher, Karlsruhe, Germany) was added at a final concentration of 483 μM (0.2 mg/mL) and cells were further incubated for 1 h at 37 °C and 5% CO_2. Afterwards, an acidified isopropanol solubilization solution containing 0.04 M hydrochloric acid and 0.1% NP-40 detergent was added to each well to lysate cells and dissolve the insoluble purple formazan

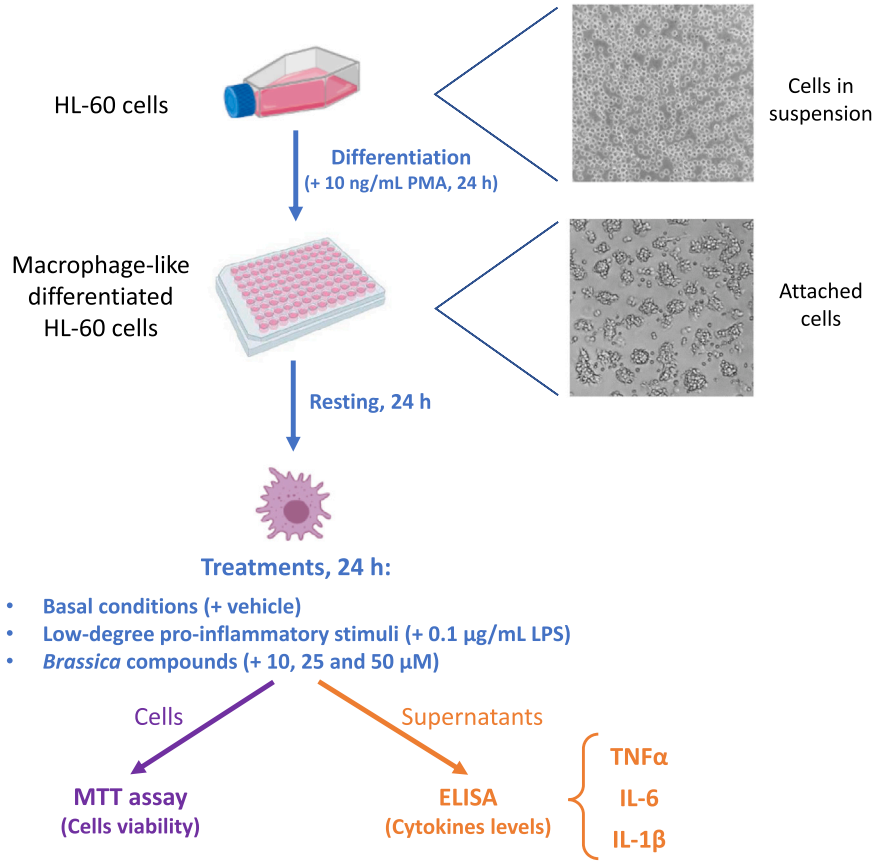


Fig. 1. Schematic representation of the protocol followed for *in vitro* cell assays. This protocol was followed first for the differentiation of HL-60 cells to active human macrophage-like cells, and then for the testing of the *Brassica* compounds' effect upon the viability of the cells (by MTT assay) and the production of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β (by ELISA).

product formed inside the cells, giving a purple-colored solution. Finally, the absorbance was measured at 550 nm by a plate-reading spectrophotometer (Spectrostar Nano; BMG Labtech, Ortenberg, Germany). Viability of treated cells was determined by comparison with control conditions (100% viability, 0% cytotoxicity), in which cells were exposed to just CCM with an equivalent dose of vehicle (DMSO). A total of three different experiments (N = 3) with three replicates for each assay condition (ten different compounds at three different doses for 24 h) were performed and analyzed for the determination of human macrophage-like cells viability.

2.7. Statistical analysis

Data are reported as normalized mean ± SEM, using the negative control as the reference level. Statistical differences were analyzed by Student t and Anova tests. Values of p under 0.05 were considered to show statistical significance. Calculations were performed using GraphPad Prism 9 and SPSS 21.0 software.

3. Results

3.1. Effect of Brassica compounds on the viability of macrophage-like HL-60 cells

The treatment of macrophage-like differentiated HL-60 cells with the tested *Brassica* compounds in this pro-inflammatory *in vitro* human macrophage-like cell model did not show any significant effect upon the viability of these cells at any of the doses (10 μM, 25 μM and 50 μM), with the only exception of SFN at the highest tested dose (50 μM), which produced a small but significant reduction on cell's viability/metabolism activity of 30% (70% cell viability/metabolism activity compared to control conditions) (Table 2).

Results represent viability (%) as the mean ± SEM following 24 h exposure of HL-60 differentiated cells to the *Brassica* compounds at the different doses indicated plus LPS (0.1 μg/mL). Data obtained from three different MTT assays (N = 3) in which three replicates were performed for each different assay condition. *p < 0.05. CQA, Chlorogenic acid (5-Caffeoyl-quinic acid); Cy3glsc, Cyanidin-3-glucoside; DIM, 3,3′-Diindoylmethane; GBS, Glucobrassicin; GER, Glucoraphenin; GRA: Glucoraphanin; GRE, Glucoerucin; I3C, Indole-3-Carbinol; SIA, Sinapic acid (Sinapinic acid); SFN, sulforaphane.

3.2. Analysis of the anti-inflammatory potential of GSLs and ITCs

The ITC SFN was the most active anti-inflammatory compound among all compounds tested (Fig. 2A). SFN was able to completely block the significant increase of LPS-induced secretion of pro-inflammatory cytokine TNF-α with all tested doses (10 μM, 25 μM and 50 μM) after 24 h treatment, reducing TNF-α production to similar levels of those observed in basal conditions. Similarly, significantly higher LPS-induced levels of IL-6 were also reduced by all SFN doses, but in this case the level of this cytokine was reduced even further with the higher SFN doses, showing a production of IL-6 below the basal conditions' levels of non-stimulated cells (Fig. 2B). The remarkable effect shown by SFN at 50 μM upon both TNF-α and IL-6 would be in part accounted to the slight reduction in cells' viability/metabolic ratio observed (~30%) with this high dose (Table 2). In the case of IL-1β, the release of this cytokine was already considerable high after cells differentiation, since the differentiation process is concomitant to the activation of macrophage-like functions, and, thus, no further increase on this cytokine level was observed following the pro-inflammatory LPS stimulus designed to mimic a low-degree inflammatory status in this human macrophage-like cell model. Nevertheless, SFN was still able to significantly reduce the production of IL-1β with the lowest dose of 10 μM, and similar reductions were observed with 25 μM and 50 μM doses, although statistical significance was not reached due to the variability of results found with the higher doses (Fig. 2C).

Among the series of aliphatic GSLs, Glucoraphanin (GRA) and Glucoraphenin (GRE) were able to reduce TNF-α production at the highest treatment dose (50 μM), while Glucoerucin (GER) showed a similar trend, but had no significant effect on the secretion of this pro-inflammatory cytokine (Fig. 3A). With regards to IL-6, GRA showed a significant reduction of this cytokine both at 25 μM (similar to basal levels) and 50 μM doses. GRE and GER were also able to significantly decrease the secretion of IL-6 at 50 μM (Fig. 3B). None of these three compounds showed any significant effect on IL-1β production at any of the administered doses (Fig. 3C).

In the case of the indolic compounds, Glucobrassicin (GBS) was the most active compound within this group, as it was able to significantly reduce the levels of all the pro-inflammatory cytokines measured. Thus, GBS was the only indolic compound capable to significantly reduce the secretion of TNF-α at 50 μM, while Idole-3-carbinol (I3C) and 3,3′-Diindoylmethane (DIM) had no effect on the release of this cytokine at none of the concentrations used (Fig. 4A). IL-6 levels, however, were clearly reduced to basal conditions by both GBS and DIM even at the lowest dose (10 μM); no effects on IL-6 levels were found when incubated with I3C (Fig. 4B). Like it occurred with TNF-α, IL-1β production levels were only reduced by GBS, but not by I3C nor DIM. However, in this case the statistically significant reduction in the production of this cytokine was observed with all GBS doses, reaching reduction IL-1β levels similar to SFN (Fig. 4C).

Table 2
Effect of *Brassica* compounds upon the viability of macrophage-like HL-60 differentiated cells under low degree inflammatory conditions.

Compound	Viability (%) Dose (μM)		
	10	25	50
GRA	100.6 ± 7.8	96.2 ± 5.0	98.7 ± 11.5
GRE	95.0 ± 5.4	110.1 ± 15.1	101.3 ± 6.5
GER	95.2 ± 4.9	89.2 ± 3.8	91.2 ± 5.0
SFN	98.2 ± 7.0	87.6 ± 3.9%	**70.0 ± 10.1***
GBS	100.7 ± 10.0	97.0 ± 3.0	96.0 ± 3.6
I3C	93.6 ± 4.6	99.5 ± 3.1	95.1 ± 6.0
DIM	101.5 ± 13.1	117.7 ± 15.7	104.8 ± 7.8
CQA	113.5 ± 11.1	114.4 ± 11.9	108.7 ± 6.8
SIA	105.7 ± 6.3	96.9 ± 5.3	101.5 ± 8.9
Cy3glc	113.7 ± 14.1	108.1 ± 4.3	107.2 ± 7.4

3.3. Analysis of the anti-inflammatory potential of phenolics present in Brassica

Neither of the two hydroxycinnamic acids tested, Chlorogenic acid (CQA) and Sinapic acid (SIA), representatives of the major phenolic acids present in cruciferous foods, showed any effect on TNF-α levels (Fig. 5A). On the contrary, both compounds were able to reduce the LPS-stimulated production of IL-6 back down to basal levels. CQA significantly reduced the IL-6 secretion at both 25 μM and 50 μM doses, while SIA was able to reduce the IL-6 levels even at 10 μM (Fig. 5B). In the case of IL-1β, only SIA reached significant reductions of this cytokine with 25 μM and 50 μM doses, while CQA showed a similar but not significant effect (Fig. 5C).

Cyanidin-3-glucoside (Cy3glc), chosen as a representative type of anthocyanin from cruciferous vegetables, was able to significantly diminish TNF-α levels at 25 μM. A similar trend, albeit not significant, was observed at 50 μM (Fig. 6A). Effects of Cy3glc on IL-6 levels were significant for all tested doses, reaching basal IL-6 levels at 25 μM and 50 μM (Fig. 6B). Cy3glc also reduced IL-1β levels in a similar fashion, but significance was only reached at the higher dose, obtaining a reduction in the production of this cytokine like that of SFN at its most active dose (Fig. 6C).

A summary of the anti-inflammatory potential of the tested

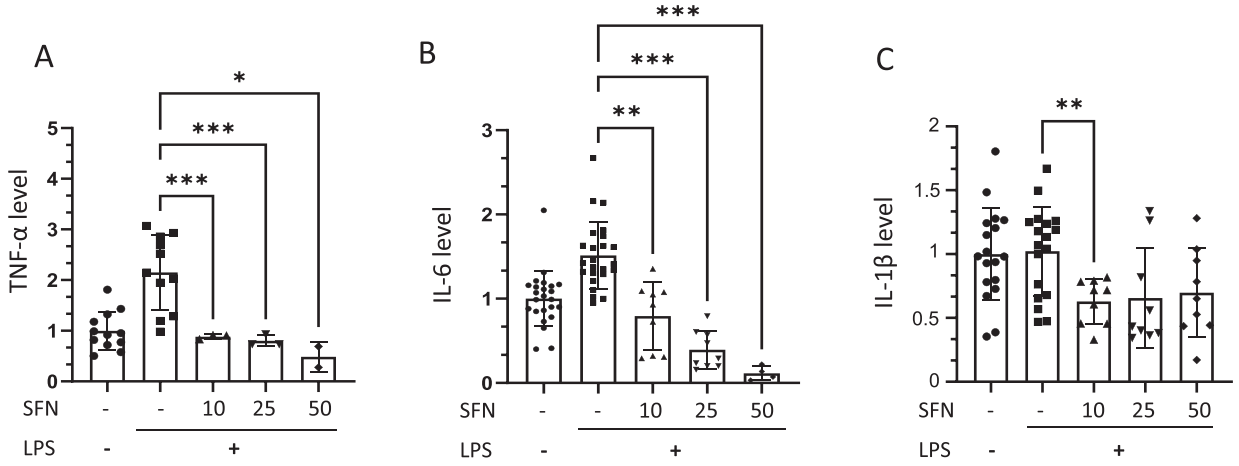


Fig. 2. Analysis of the anti-inflammatory potential of SFN. Bar diagrams represent the normalized mean ± SEM levels of TNF-α (**A**), IL-6 (**B**) and IL-1β (**C**) following different treatments for 24 h: No treatment (negative control); LPS treatment alone (0.1 μg/mL; positive pro-inflammatory control); and different doses of **SFN** (10, 15 and 50 μM) in the presence of LPS at 0.1 μg/mL. Dot-plots represent individual data from at least 3 different assays (N = 2–21) with the different assay conditions. Statistically significant differences from are represented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Although it is not graphically represented for simplification, LPS-induced levels of TNF-α (**A**) and IL-6 (**B**), but not IL-1β (**C**), were significantly increase ($p < 0.05$).

compounds is shown in Table 3. SFN, GBS and Cy3glc were the most active compounds, as they were able to reduce the release of all the pro-inflammatory cytokines tested by human macrophage-like HL-60 cells after LPS treatment. In addition, the aliphatic GSLs GRA and GRE also exhibited an important anti-inflammatory activity *in vitro*, as they were able to reduce the production of both TNF-α and IL-6. The SIA was also able to reduce the production of two out of the three analyzed cytokines, in this case IL-6 and IL-1β. Other *Brassica* compounds such as DIM, CQA and GER were also able to exclusively lower the IL-6 levels. I3C was the only tested compound that failed to show any anti-inflammatory potential in our human cell-line *in vitro* model (Table 3).

The plus sign (+) represents positive anti-inflammatory potential of compound, while negative sign (-) indicates no anti-inflammatory effect. Thus, + means that at least one of the tested doses inhibit the production of the indicated cytokine, ++ indicate that inhibition occurs with two of the doses, and +++ indicate that the compound was able to inhibit the cytokine production with all tested doses. CQA, Chlorogenic acid (5-Caffeoyl-quinic acid); Cy3glsc, Cyanidin-3-glucoside; DIM, 3,3′-Diindoylmethane; GBS, Glucobrassicin; GER, Glucoraphenin; GRA: Glucoraphanin; GRE, Glucoerucin; I3C, Indole-3-Carbinol; IL-1β, interleukin 1 beta; IL-6, interleukin 6; SIA, Sinapic acid (Sinapinic acid); SFN, sulforaphane; TNF-α, tumor necrosis factor alpha.

4. Discussion

In this study, we have evaluated the anti-inflammatory potential of a series of *Brassica* compounds in *an in vitro* model of human macrophage-like cells subjected to a low-degree inflammatory scenario, finding that indeed most of the tested compounds presented anti-inflammatory potential, as they were able to reduce the secretion of key pro-inflammatory cytokines TNF-α, IL-6 and IL-1β. These data confirm the possibility of using several *Brassica* compounds to prevent or treat inflammatory chronic diseases and provide an orientation for further research in which *Brassica* enriched in bioactive compounds (GSL/ITC and phenolics) could be used as natural cocktails of health-promoting bioactive compounds or be the natural source of single bioactive compounds that may be used to develop new anti-inflammatory therapies.

Initially, we have established an *in vitro* model based on the differentiation of the human leukemia cell line HL-60 into macrophage-like cells to analyze the anti-inflammatory activity of a set of phytochemicals abundant in *Brassica* vegetables. In this model, cells were first differentiated to macrophage-like cells, and then subjected to a 'light' pro-inflammatory condition, to finally analyze the anti-inflammatory potential of the studied compounds. Thus, with this human *in vitro* cell model we were able to mimic the low-degree inflammatory scenario present in clinically relevant chronic inflammatory diseases, to test the anti-inflammatory potential of the phytochemicals. Specifically, the macrophage-like cells were obtained by differentiation of HL-60 cells with PMA. Differentiated cells were then subjected to 24 h treatment with LPS at 0.1 μg/mL, as a low-degree pro-inflammatory stimulus, in the presence or absence of the tested compounds at different doses. The viability of the cells, determined by MTT assays, and the anti-inflammatory potential of the compounds was finally measured as relative reduction in the production and release of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β by using ELISA assays (Fig. 1).

Most of the tested compounds present in *Brassica* showed no effect upon the viability of differentiated HL-60 macrophage-like cells, with the only exception of the slight cytostatic effect caused by SFN at the highest tested dose (50 μM), while almost all of them showed anti-inflammatory activity, with the only exception of Idole-3-carbinol (I3C) (Table 3). In particular, the levels of IL-6, which is key in the inflammatory process found in inflammatory pathologies, were remarkably reduced by the tested phytochemicals as the release of this cytokine by the differentiated macrophage-like cells was reduced by almost all the active compounds (nine out of ten).

Among the active compounds, SFN, GBS and Cy3glc showed the highest anti-inflammatory activities, as these three biomolecules were able to reduce the production of all the analyzed cytokines (TNF-α, IL-6 and IL-1β). IL-6 levels were markedly reduced by these three active compounds at all tested doses, even with the lowest one (10 μM). Furthermore, SFN was also capable to inhibit TNF-α production at all tested doses, while all GBS doses tested did the same with IL-1β. In the case of IL-1β, it is important to note that the release of this cytokine was already elevated in this human macrophage-like cell model after cell differentiation, as this differentiation process is accompanied by the activation of the macrophage-like functions of these cells (including cytokine production) and, therefore, no further increase on this cytokine level was observed following a low-dose pro-inflammatory LPS stimulus designed to mimic a low-degree endotoxemia typical of chronic diseases like obesity and related metabolic disorders rather than an acute intense response. Nonetheless, the ability of these compounds to efficiently reduce the production of these three pro-inflammatory cytokines, specially of IL-6, remarks their anti-inflammatory potential, as these cytokines produced by macrophages play a major role in the

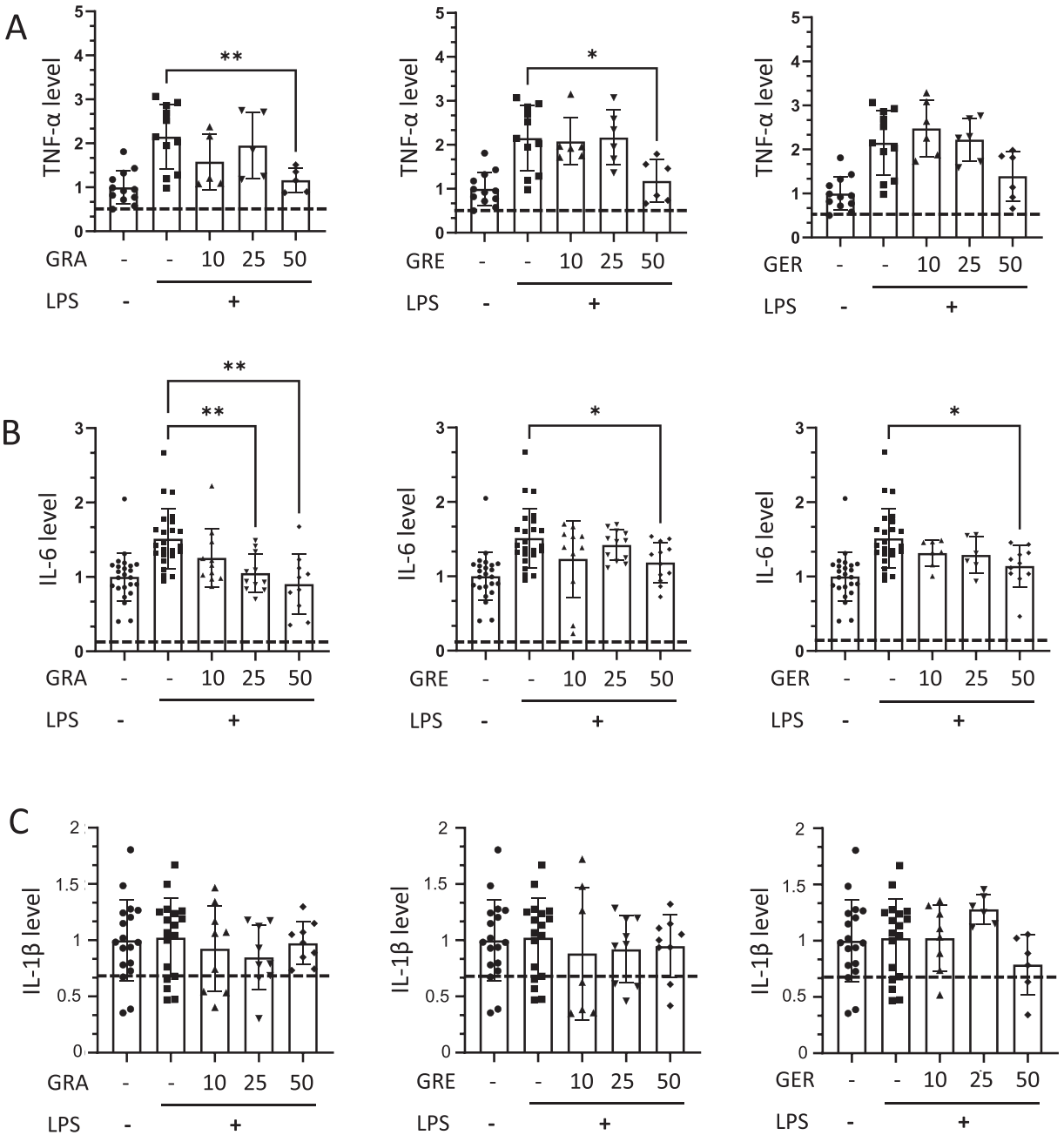


Fig. 3. Analysis of the anti-inflammatory potential of aliphatic GSLs. Bar diagrams represent the normalized mean ± SEM levels of TNF-α (**A**), IL-6 (**B**) and IL-1β (**C**) following different treatments for 24 h: No treatment (negative control); LPS treatment alone (0.1 μg/mL; positive pro-inflammatory control); and different doses of **GRA**, **GRE** or **GER** (10, 15 and 50 μM) + LPS (0.1 μg/mL). Dot-plots represent individual data from at least 3 different assays (N = 5–21) with the different assay conditions. Statistically significant differences from are represented as *$p < 0.05$; **$p < 0.01$. Although it is not graphically represented for simplification, LPS-induced levels of TNF-α (**A**) and IL-6 (**B**), but not IL-1β (**C**), were significantly increase ($p < 0.05$). Dash line displays internal positive control, which represents the level of the corresponding cytokine following treatment with the most active dose of SFN without affected cell viability (25uM).

establishment and maintenance of chronic inflammation related diseases such as cirrhosis [35,36].

Our data confirm the anti-inflammatory potency of SFN, as our results in this particular human *in vitro* model correlate with those from other authors obtained in other cell models, such as murine macrophages [37], as well as with those that observed the capacity of SFN to negatively regulate the pro-inflammatory effect of LPS by inhibiting TLR4-CD14 derived cell activation [34,38], and block the downstream NFκB cell signaling pathway activation and the subsequent transcription of pro-inflammatory cytokines genes [39].

To the best of our knowledge, this is the first study reporting a remarkable anti-inflammatory activity of GBS *in vitro*, as it was able to inhibit the production of all the pro-inflammatory cytokines analyzed herein. Meanwhile, the GBS autolysis derivative DIM, which has been shown to be an anti-inflammatory agent in other *in vitro* and *in vivo* models [40], was found to be less active than GBS in our human macrophage-like cell model, since DIM was only able to notably inhibit IL-6 production, but not TNF-α or IL-1β. On the contrary, I3C, the other natural hydrolysis product of GBS tested, did not show any anti-inflammatory activity, as it was unable to alter the level of any of the tested cytokines.

The third most active anti-inflammatory compound found in our

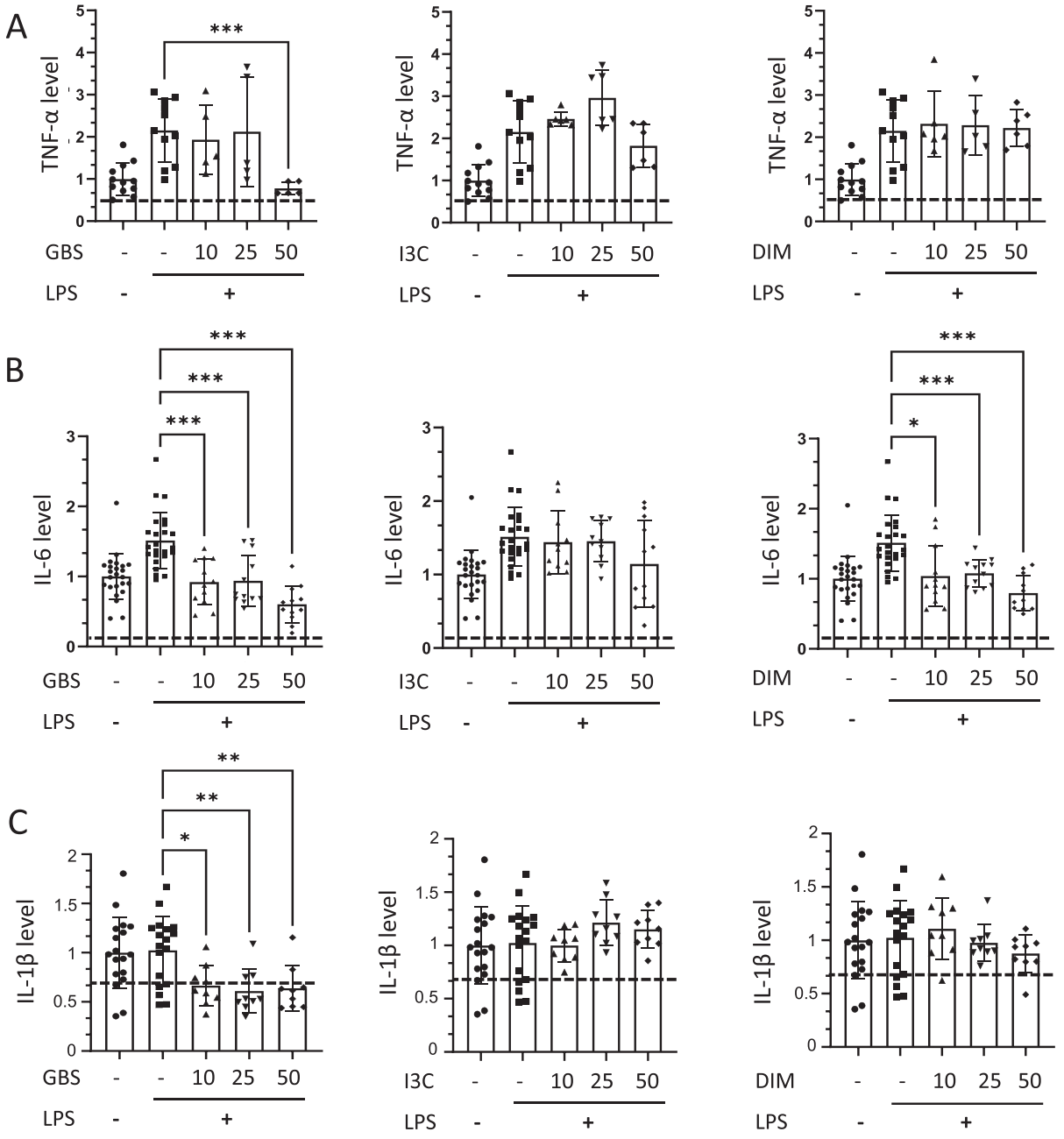


Fig. 4. Analysis of the anti-inflammatory potential of indolic compounds. Bar diagrams represent the normalized mean ± SEM levels of TNF-α (**A**), IL-6 (**B**) and IL-1β (**C**) following different treatments for 24 h: No treatment (negative control); LPS treatment alone (0.1 μg/mL; positive pro-inflammatory control); and different doses of **GBS**, **I3C** or **DIM** (10, 15 and 50 μM) + LPS (0.1 μg/mL). Dot-plots represent individual data from at least 3 different assays (N = 5–21) with the different assay conditions. Statistically significant differences from are represented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Although it is not graphically represented for simplification, LPS-induced levels of TNF-α (**A**) and IL-6 (**B**), but not IL-1β (**C**), were significantly increase ($p < 0.05$). Dash line displays internal positive control, which represents the level of the corresponding cytokine following treatment with the most active dose of SFN.

human macrophage-like *in vitro* model was Cy3glc, as it not only clearly inhibited IL-6 secretion even at the lowest dose (10 μM), but also it was able to reduce TNF-α and IL-1β levels when cells were treated with higher doses. Although supportive data on the down-regulation of TNF-α and IL-6 mRNA produced by Cy3glc was already shown in a N9 microglia cell line [41], this is the first time that the direct anti-inflammatory activity of Cy3glc upon the release of pro-inflammatory cytokines has been demonstrated in an *in vitro* model of human macrophages. Our data on the anti-inflammatory activity of this anthocyanin are in concordance and complementary with data from other authors, who have shown how Cy3glc, but also a mixture of these compounds and related metabolites, were able to reduce inflammation induced adhesion of THP-1 cells, another myeloid human cell line [42]. Altogether, these data remark the interest of studying anthocyanins as potentially relevant anti-inflammatory agents and the cruciferous vegetables rich in these compounds (such as the red radish or red cabbages, as well as other sprouts and micro-vegetables from cruciferous origin, rich in anthocyanins) as the natural source of these bioactive compounds.

Both hydroxycinnamic acids tested, CQA and SIA, showed anti-inflammatory activity, but while CQA was only able to inhibit IL-6 production with the high doses (25 μM and 50 μM), SIA was also able to inhibit IL-1β with the same doses and to potently inhibit IL-6 even

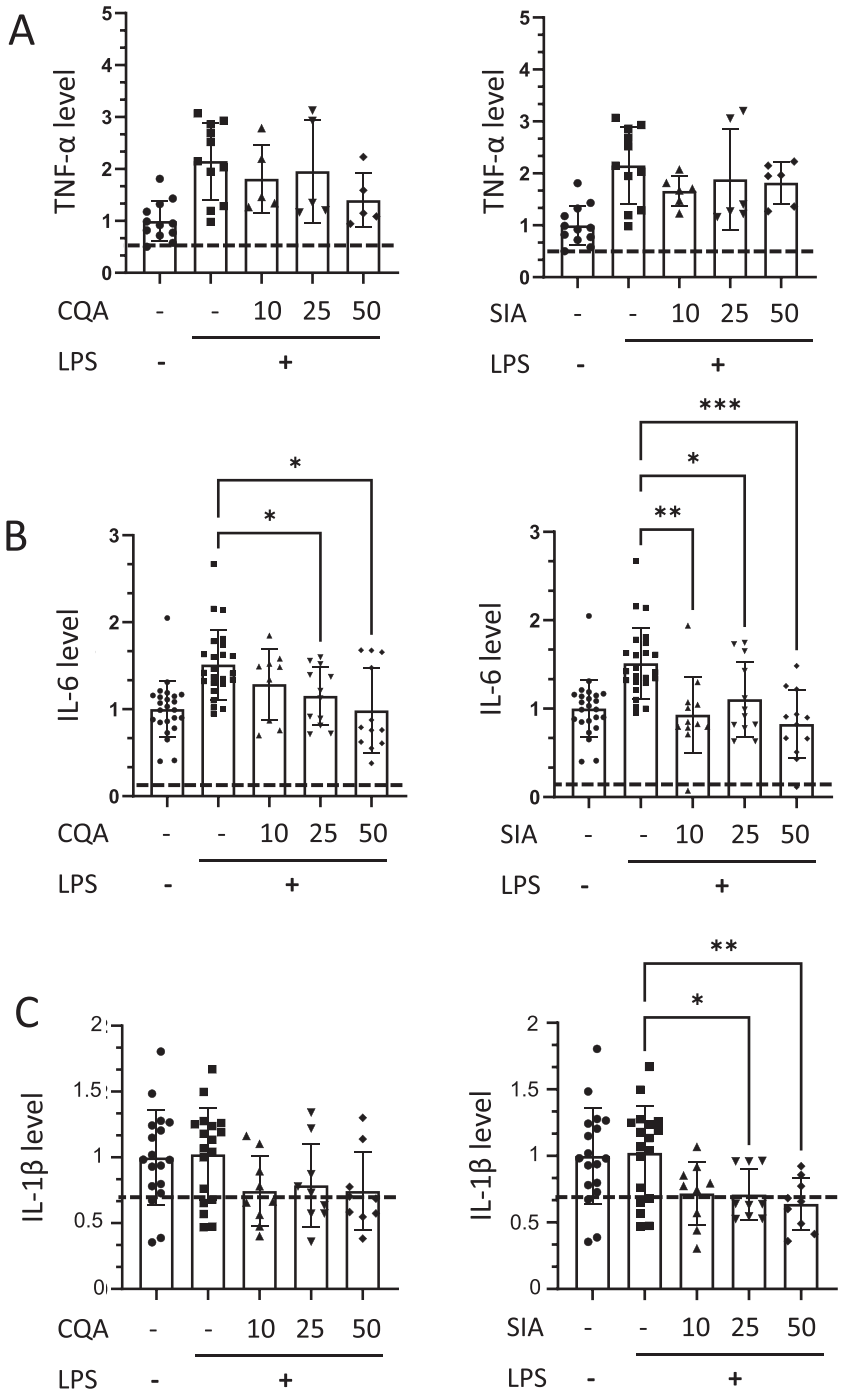


Fig. 5. Analysis of the anti-inflammatory potential of hydroxycinnamic acids. Bar diagrams represent the normalized mean ± SEM levels of TNF-α (**A**), IL-6 (**B**) and IL-1β (**C**) following different treatments for 24 h: No treatment (negative control); LPS treatment alone (0.1 μg/mL; positive pro-inflammatory control); and different doses of **CQA** or **SIA** (10, 15 and 50 μM) + LPS (0.1 μg/mL). Dot-plots represent individual data from at least 3 different assays (N = 5–21) with the different assay conditions. Statistically significant differences from are represented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Although it is not graphically represented for simplification, LPS-induced levels of TNF-α (**A**) and IL-6 (**B**), but not IL-1β (**C**), were significantly increase ($p < 0.05$). Dash line displays internal positive control, which represents the level of the corresponding cytokine following treatment with the most active dose of SFN.

with the lowest dose (10 μM). Our data on CQA and SIA anti-inflammatory activity in this human macrophage-like HL-60 derived model agree with others' results obtained from different *in vitro* and *in vivo* models [43,44].

Finally, the aliphatic GSLs, GRA, GRE and GER, that are present in high amounts in cruciferous foods (*e.g.*, broccoli, radish, rocket salad, *etc.*) have been previously shown to exert anti-inflammatory effects through their conversion into their derivative SFN by the myrosinase enzyme present in plants [45]. Interestingly, here we have been able to show how these three compounds present anti-inflammatory potential by themselves. Thus, when used at maximum dose (50 μM) they were able to reduce IL-6 and TNF-α production *in vitro*, with the only exception of GER, which showed the same trend, but did not reach a significant reduction of TNF-α.

According to the results presented in this study, it is demonstrated that the analyzed phytochemicals might act separately as single anti-inflammatory agents when used at low concentrations (in the micromolar range), compatible with those expected to have a physiological relevance. These data also confirm that the potential health-promoting effect of bioactive compounds requires to consider the complexity of the natural food matrix where those compounds are present. The cruciferous foods (*Brassicaceae*) are rich in bioactive compounds (GSL, and their cognate isothiocyanates; hydroxycinnamic acids derivatives; and flavonols, among others) that would be delivered through diet. Therefore, the control of the factors that affect the composition of the food or food product to deliver the necessary amount of bioactive

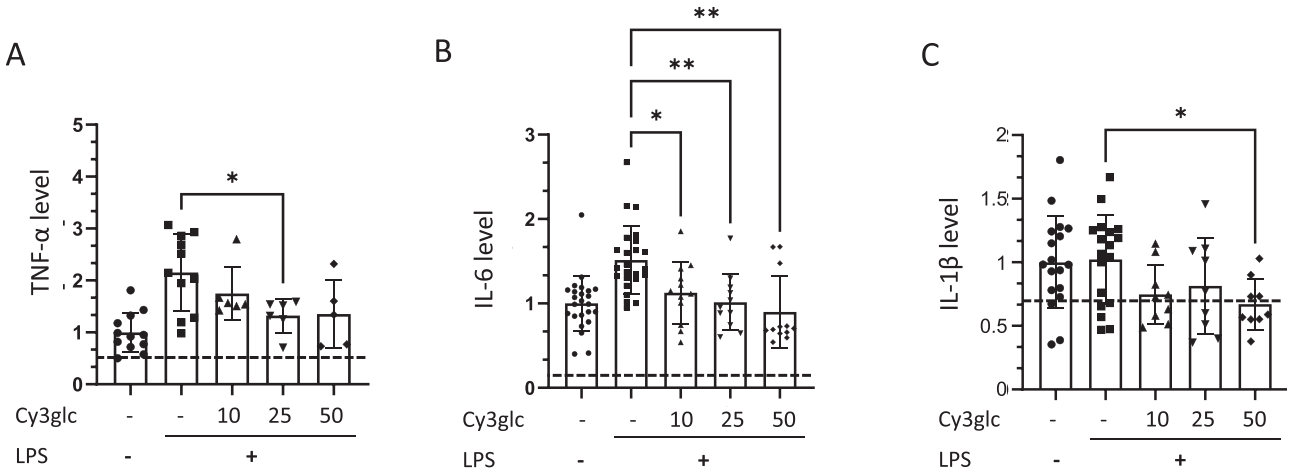


Fig. 6. Analysis of the anti-inflammatory potential of cyanidin-3-glucoside. Bar diagrams represent the normalized mean ± SEM levels of TNF-α (**A**), IL-6 (**B**) and IL-1β (**C**) following different treatments for 24 h: No treatment (negative control); LPS treatment alone (0.1 μg/mL; positive pro-inflammatory control); and different doses of **Cy3glc** (10, 15 and 50 μM) + LPS (0.1 μg/mL). Dot-plots represent individual data from at least 3 different assays (N = 5–21) with the different assay conditions. Statistically significant differences from are represented as *$p < 0.05$; **$p < 0.01$. Although it is not graphically represented for simplification, LPS-induced levels of TNF-α (**A**) and IL-6 (**B**), but not IL-1β (**C**), were significantly increase ($p < 0.05$). Dash line displays internal positive control, which represents the level of the corresponding cytokine following treatment with the most active dose of SFN.

Table 3
Summary of the anti-inflammatory potential of tested compounds.

	SFN	GRA	GRE	GER	CQA	SIA	Cy3glc	GBS	DIM	I3C
TNF-α	+++	+	+	–	–	–	+	+	–	–
IL-6	+++	++	+	+	+	+++	+++	+++	+++	–
IL-1β	+	–	–	–	–	++	+	+++	–	–

compounds is crucial [19,20]. In this context, previous studies were focused either in one pure compound, or an individual food from which normally only a single bioactive compound was characterized or followed (*e.g.*, SFN) and classically higher doses were used to test their biological activity.

In this work, the anti-inflammatory activity of not only one, but a series of *Brassica* phytochemicals, showed strong evidence for the potential protective anti-inflammatory effects of both, the parental compounds, and their metabolites (*e.g.*, GSLs and ITCs), even at low doses like those that might reach human macrophages resident in tissues under physiological conditions.

Therefore, for the design of nutritional interventions, it is crucial to fully characterize the food or food product (natural matrix rich in bioactive compounds), to know exactly the specific dosage and administration, together with the pattern of consumption, in order to find the relationships between the intake of the given phytochemical and/or food, and the expected health-promoting outcome.

This point is of great relevance in the development of dietary recommendations for the prevention or amelioration of the pathological situation of patients suffering of chronic inflammatory diseases such as endometriosis [46], among many others [47]. Thus, further research involving cruciferous sprouts (very rich in GSLs, hydroxycinnamic acids and anthocyanins) will be carried out to further explore the potential functionalities of these vegetables and their bioactive compounds beyond the cells and animals' models and into the human studies in the clinic.

In conclusion, most of the *Brassica*'s phytochemicals tested herein are demonstrated to be potential natural anti-inflammatory agents, as most of them can reduce, at different levels, the production of pro-inflammatory cytokines that are key for the establishment and maintenance of systemic, long-term inflammatory processes that cause numerous chronic pathologies. Thus, these results highlight the potential utility of these compounds and their vegetables of origin as anti-inflammatory agents that may be an important approach to reduce the global health issues related to inflammatory pathologies. In this regard, an optimal nutritional intervention in which the proper agents and doses are considered will be of great use for the clinical managing of those pathologies.

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CRediT authorship contribution statement

Conceptualization, A.J.R.-A., M.A.M.-S., P.G.-P., M.M.-E., B.R.-M. and D.A.M.; Methodology, A.J.R.-A., B.R.-M. and D.A.M.; Software, A. J.R.-A., M.A.M.-S., P.G.-P., M.M.-E., B.R.-M. and D.A.M.; Validation, A.J.R.-A., M.A.M.-S., P.G.-P., M.M.-E., B.R.-M. and D.A.M.; Formal analysis, A.J.R.-A., B.R.-M. and D.A.M.; Investigation, A.J.R.-A., M.A.M.-S., B.R.-M. and D.A.M.; Resources, A.J.R.-A., M.A.M.-S., P.G.-P., M.M.-E., B.R.-M. and D.A.M.; Data curation, A.J.R.-A., M.A.M.-S., P.G.-P., M. M.-E., B.R.-M. and D.A.M.; Writing – original draft preparation, A.J.R.-A., B.R.-M. and D.A.-M.; Writing – review & editing, A.J.R.-A., M.A. M.-S., P.G.-P., M.M.-E., B.R.-M. and D.A.M.; Visualization, A.J.R.-A., M. A.M.-S., P.G.-P., M.M.-E., B.R.-M. and D.A.M.; Supervision, A.J.R.-A., B. R.-M. and D.A.M.; Project administration, A.J.R.-A., B.R.-M. and D.A.-

M.; Funding acquisition, A.J.R.-A., P.G.-P., M.M.-E., B.R.-M. and D.A.-M. All authors have read and agreed to the published version of the manuscript.

Conflict of interest statement

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Authors' contributions

A.J.R.A. conceived and designed the experimental plan, performed experiments, analyzed the data, and wrote the manuscript. M.A.M. performed experiments and wrote the manuscript. P.G.P. provided the necessary facilities and wrote the manuscript. M.M.E. wrote the manuscript. B.R.M. conceived and designed the experimental plan, performed experiments, analyzed the data, and wrote the manuscript. D.A.M. conceived and designed the experimental plan, provided vegetal compounds, and wrote the manuscript. All authors read and approved the manuscript.

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