

Short Communication

Dihydroxylated phenolic acids derived from microbial metabolism reduce lipopolysaccharide-stimulated cytokine secretion by human peripheral blood mononuclear cells

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Oligomers and polymers of flavan-3-ols (proanthocyanidins) are very abundant in the Mediterranean diet, but are poorly absorbed. However, when these polyphenols reach the colon, they are metabolised by the intestinal microbiota into various phenolic acids, including phenylpropionic, phenylacetic and benzoic acid derivatives. Since the biological properties of these metabolites are not completely known, in the present study, we investigated the effect of the following microbial phenolic metabolites: 3,4-dihydroxyphenylpropionic acid (3,4-DHPPA), 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), 3-hydroxyphenylacetic acid, 4-hydroxybenzoic acid and 4-hydroxyhippuric acid (4-HHA), on modulation of the production of the main pro-inflammatory cytokines (TNF- α , IL-1 β and IL-6). The production of these cytokines by lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) pre-treated with the phenolic metabolites was studied in six healthy volunteers. With the exception of 4-HHA for TNF- α secretion, only the dihydroxylated compounds, 3,4-DHPPA and 3,4-DHPAA, significantly inhibited the secretion of these pro-inflammatory cytokines in LPS-stimulated PBMC. Mean inhibition of the secretion of TNF- α by 3,4-DHPPA and 3,4-DHPAA was 84.9 and 86.4 %, respectively. The concentrations of IL-6 in the culture supernatant were reduced by 88.8 and 92.3 % with 3,4-DHPPA and 3,4-DHPAA pre-treatment, respectively. Finally, inhibition was slightly higher for IL-1 β , 93.1 % by 3,4-DHPPA and 97.9 % by 3,4-DHPAA. These results indicate that dihydroxylated phenolic acids derived from microbial metabolism present marked anti-inflammatory properties, providing additional information about the health benefits of dietary polyphenols and their potential value as therapeutic agents.

TNF- α : IL-1 β : IL-6: Atherosclerosis: Phenolic acids: Microbial metabolism

Polyphenols are among the most abundant antioxidant compounds of the Mediterranean diet and may play a key role in the prevention of cardiovascular and neurodegenerative diseases, and cancer⁽¹⁾. Health effects derived from polyphenol consumption depend on their bioavailability, a factor that greatly varies from one compound to another⁽²⁾. Among polyphenols, the oligomers and polymers of flavan-3-ols, also called proanthocyanidins, are not absorbed or degraded into

monomers during their transit through the stomach^(2,3). However, when they reach the colon, they are metabolised by the intestinal microbiota into various phenolic acids, including phenylpropionic, phenylacetic and benzoic acid derivatives⁽⁴⁾. Hydroxycinnamic acid esters and polyphenols linked to rhamnose are also degraded into phenolic acids by the microbiota⁽²⁾. Recently, it has been reported that these metabolites may also exert several biological activities, such

Abbreviations: 3,4-DHPAA, 3,4-dihydroxyphenylacetic acid; 3,4-DHPPA, 3,4-dihydroxyphenylpropionic acid; 3-HPAA, 3-hydroxyphenylacetic acid; 3-HPPA, 3-hydroxyphenylpropionic acid; 4-HBA, 4-hydroxybenzoic acid; 4-HHA, 4-hydroxyhippuric acid; LPS, lipopolysaccharide; PBMC, peripheral blood mononuclear cells.

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as the inhibition of platelet aggregation and activation function⁽⁵⁾, inhibition of cyclo-oxygenase-2 in HT-29 colon cancer cells⁽⁶⁾, reduction in the synthesis of prostanoids in colon cells⁽⁷⁾, antiproliferative activity in prostate and cancer cells⁽⁸⁾ and, finally, influence cell proliferation, apoptosis and signalling pathways in human colon carcinoma cells⁽⁹⁾.

Atherosclerosis is now considered to be a low-grade chronic inflammatory process resulting from the interactions between plasma lipoproteins, cellular components (monocyte/macrophages, T lymphocytes, endothelial cells and smooth muscle cells) and the extracellular matrix of arterial wall⁽¹⁰⁾. Pro-inflammatory cytokines are involved in all phases of the atherosclerotic process: they stimulate chemokines and adhesion molecules, leading to early recruitment of monocytes and lymphocytes in the arterial intima, and later exert potential noxious effects promoting weakening of plaques that are more prone to rupture⁽¹⁰⁾. However, data concerning the effect of phenolic compounds on the production of the inflammatory mediators from mononuclear cells are scarce and contradictory. Some researchers⁽¹¹⁾ reported that polyphenols from cocoa may reduce the expression of IL-2 mRNA in human lymphocytes, and others⁽¹²⁾ found differential effects of isolated cocoa procyanidin fractions (monomer to decamers) on the expression and secretion of IL-1 β from peripheral blood mononuclear cells (PBMC). In the same way, cocoa polyphenols produced an increase in TNF- α secretion⁽¹³⁾ or even a down-regulation of IL-2 secretion and IL-2 receptor surface expression on a lymphoid cell line⁽¹⁴⁾, whereas in another study, polyphenols from olive oil had no effect on the secretion of TNF- α , IL-1 β or IL-6 in the human whole blood⁽¹⁵⁾.

Considering the lack of information regarding the anti-inflammatory properties of microbial-derived phenolic acids, the aim of the present study was to investigate the effect of some microbial phenolic metabolites on the modulation of the production of the most representative pro-inflammatory cytokines, i.e. TNF- α , IL-1 β and IL-6, in lipopolysaccharide (LPS)-stimulated PBMC from healthy human volunteers.

Methods

Six healthy volunteers (two men and four women) with an average age of 29.3 (SD 2.1) years (range 27–33 years), weight of 62.8 (SD 18.5) kg (range 50–100 kg), height of 1.7 (SD 0.1) m (range 1.6–1.9 m) and BMI of 21.5 kg/m² (SD 3.4) (range 17.9–27.7 kg/m²) participated in the study. None of them reported a history of heart disease, homeostatic disorder or any other medical disease. None were receiving any medication or taking any vitamin supplements. A 24 h food recall questionnaire was used to assess their habitual nutrient intake. This information was converted into dietary data using the Professional Diet Balancer software (Cardinal Health Systems, Inc., Edina, MN, USA). Their habitual diet (mean of six volunteers) included an intake of 9789.81 kJ/d (2339.82 kcal/d); 117.41 g/d protein; 257.35 g/d carbohydrates; 24.99 g/d dietary fibre; 5.27 g/d soluble fibre; 76.43 g/d total sugar (14.93 g/d monosaccharides, 31.27 g/d disaccharides); 92.48 g/d of total fat (26.03 g/d saturated fat, 43.30 g/d monounsaturated fat, 15.93 g/d polyunsaturated fat, 1.22 g/d *trans*-fatty acids, 221.87 mg/d cholesterol); 863.77 retinol equivalents/d vitamin A; 2.33 mg/d vitamin

B₁; 2.39 mg/d vitamin B₂; 40.79 mg/d vitamin B₃; 2.49 mg/d vitamin B₆; 7.77 μ g/d vitamin B₁₂; 84.91 mg/d vitamin C; 4.89 μ g/d vitamin D; 13.08 mg/d vitamin E; 144.47 mg/d estimated polyphenol intake; 181.65 mg/d phytoesterols.

Peripheral blood from the volunteers was collected and PBMC were isolated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden)⁽¹⁶⁾. Harvested cells were washed with PBS 10 \times buffer (Roche Diagnostics GmbH, Mannheim, Germany) and then counted in a haemocytometer chamber. Cell viability was estimated with trypan blue. PBMC were resuspended in RPMI-1640 (Biowhittaker, Verviers, Belgium) containing fetal bovine serum (10%) and gentamicin (0.05 mg/ml) (RPMI-10% fetal) up to a concentration of 1×10^6 viable cells/ml.

For each of the following phenolic acids, a 3 μ M solution was prepared in RPMI-10% fetal: 3,4-dihydroxyphenylpropionic acid (3,4-DHPPA); 3-hydroxyphenylpropionic acid (3-HPPA); 3,4-dihydroxyphenylacetic acid (3,4-DHPAA); 3-hydroxyphenylacetic acid (3-HPAA); 4-hydroxybenzoic acid (4-HBA; Sigma-Aldrich, St Louis, MO, USA); 4-hydroxyhippuric acid (4-HHA; PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany).

PBMC were cultured with the different phenolic acid solutions in the presence of LPS (Sigma-Aldrich). Five hundred microlitres of the 1×10^6 cells/ml suspension (5×10^5 cells, total number of cells) were pre-treated (16 h at 37°C, 5% CO₂) with 250 μ l of the 3 μ M-phenolic acid solution (1 μ M, final concentration with cells) in twenty-four-well plates. After the pre-treatment period, the cell viability was estimated with trypan blue and was higher than 95%. LPS (1 μ g/ml) was then added to the culture followed by incubation for 72 h at 37°C. Unstimulated and LPS-stimulated polyphenol-free cells were also cultured under the same conditions. Experiments were performed in duplicate. After the incubation period, the cultures were centrifuged and the supernatant collected and stored at -80°C until analysis. Pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α were determined in the culture supernatants by ELISA (Bender Med Systems GmbH, Vienna, Austria). Detection limits were as follows: 0.7 pg/ml for IL-1 β ; 0.92 ng/ml for IL-6; 1.65 pg/ml for TNF- α .

For the statistical treatment of the data, *t* test for paired samples were performed using the PC software package SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). Differences between values were expressed as the percentage of enhancement or inhibition. All statistical tests were two-tailed, and the significance level was 0.05.

Results

The effects of tested phenolic acids on the secretion of TNF- α , IL-1 β and IL-6 from the PBMC of healthy subjects are shown in Fig. 1 and Table 1. Stimulation of PBMC with LPS significantly increased the levels of the three pro-inflammatory cytokines up to 295.48 pg/ml (min = 48.34, max = 773.45) for TNF- α , 128.38 pg/ml (min = 44.24, max = 209.91) for IL-1 β and 386.58 pg/ml (min = 331.36, max = 475.57) for IL-6.

3,4-DHPPA, 3,4-DHPAA and 4-HHA significantly reduced ($P < 0.01$ for 3,4-DHPPA and 3,4-DHPAA, $P < 0.05$ for 4-HHA) TNF- α secretion in LPS-stimulated PBMC by 84.9, 86.4 and 30.4%, respectively (Table 1). Contrarily, 4-HBA significantly increased TNF- α secretion by 9.9%. No significant

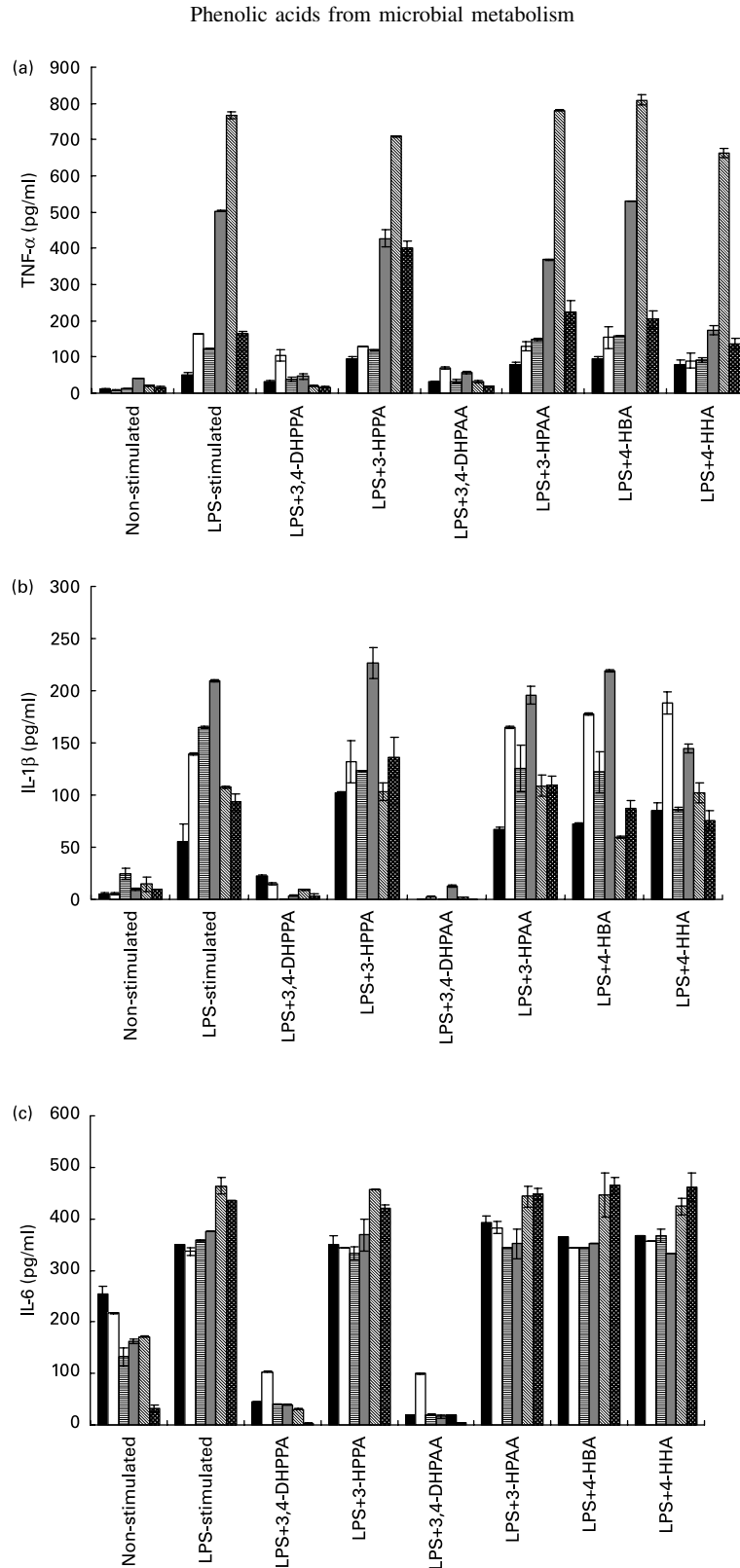


Fig. 1. Effect of phenolic acids ($1 \mu\text{M}$) derived from microbial metabolism on the secretion of (a) TNF- α , (b) IL-1 β and (c) IL-6, after stimulation with lipopolysaccharide (LPS) in peripheral blood mononuclear cells from six subjects (■, 1; □, 2; ▨, 3; ▩, 4; ▪, 5; ▫, 6). 3,4-DHPPA, 3,4-dihydroxyphenylpropionic acid; 3-HPAA, 3-hydroxyphenylpropionic acid; 3,4-DHPAA, 3,4-dihydroxyphenylacetic acid; 3-HPAA, 3-hydroxyphenylacetic acid; 4-HBA, 4-hydroxybenzoic acid; 4-HHA, 4-hydroxyhippuric acid.

changes in TNF- α secretion were recorded after the addition of the remaining tested phenolic acids, 3-HPAA and 3-HPAA. In the case of IL-1 β , only 3,4-DHPPA and 3,4-DHPAA significantly ($P < 0.001$) reduced the secretion of this cytokine in

LPS-stimulated PBMC by 93.1 and 97.9%, respectively (Fig. 1; Table 1). Similarly, a significant reduction ($P < 0.001$) in IL-6 secretion from LPS-stimulated PBMC was also observed after treatment with 3,4-DHPPA and 3,4-DHPAA, resulting in

Table 1. Summary results of the effect of phenolic acids (1 μM) derived from microbial metabolism on the secretion of TNF- α , IL-1 β and IL-6 after stimulation with lipopolysaccharide (LPS) in peripheral blood mononuclear cells from six healthy subjects

(Mean values with their standard deviations)

<i>n</i> 6	Minimum	Maximum	Mean	SD	Enhancement or inhibition (%)	<i>P</i> *
TNF-α (pg/ml)						
Non-stimulated	5.73	40.55	18.23	11.51		
LPS-stimulated	48.34	773.45	295.48	265.97		
LPS + 3,4-DHPPA	16.55	128.15	44.51	35.66	-84.9	0.009
LPS + 3-HPPA	87.07	709.68	313.14	232.76	+6.0	0.588
LPS + 3,4-DHPAA	17.95	72.51	40.06	18.35	-86.4	0.007
LPS + 3-HPAA	76.07	781.47	288.48	249.69	-2.4	0.728
LPS + 4-HBA	88.51	819.10	324.87	270.58	+9.9	0.001
LPS + 4-HHA	69.86	672.04	205.67	216.82	-30.4	0.026
IL-1β (pg/ml)						
Non-stimulated	4.36	28.12	11.39	7.41		
LPS-stimulated	44.24	209.91	128.38	52.60		
LPS + 3,4-DHPPA	0.00	22.28	8.85	7.94	-93.1	0.000
LPS + 3-HPPA	97.49	236.79	138.57	46.73	+10.5	0.192
LPS + 3,4-DHPAA	0.00	12.00	2.72	4.65	-97.9	0.000
LPS + 3-HPAA	65.05	202.09	128.54	44.20	+0.1	0.982
LPS + 4-HBA	59.15	218.71	122.92	60.68	-4.3	0.583
LPS + 4-HHA	68.54	195.74	113.70	42.28	-11.4	0.323
IL-6 (pg/ml)						
Non-stimulated	27.51	264.91	161.22	73.60		
LPS-stimulated	331.36	475.57	386.58	49.38		
LPS + 3,4-DHPPA	2.94	102.68	43.29	31.14	-88.8	0.000
LPS + 3-HPPA	324.35	456.42	378.79	48.03	-2.0	0.138
LPS + 3,4-DHPAA	2.94	99.42	29.59	33.18	-92.3	0.000
LPS + 3-HPAA	331.36	458.06	393.83	44.24	+1.9	0.482
LPS + 4-HBA	343.01	476.96	386.05	54.08	-0.1	0.946
LPS + 4-HHA	333.34	480.36	385.20	46.68	-0.4	0.880

3,4-DHPPA, 3,4-dihydroxyphenylpropionic acid; 3-HPPA, 3-hydroxyphenylpropionic acid; 3,4-DHPAA, 3,4-dihydroxyphenylacetic acid; 3-HPAA, 3-hydroxyphenylacetic acid; 4-HBA, 4-hydroxybenzoic acid; 4-HHA, 4-hydroxyhippuric acid.

* Significant differences between LPS-stimulated cells and those in the presence of phenolic acids were determined by the *t* test.

an inhibition of 88.8 and 92.3 %, respectively. However, no significant changes were found in IL-1 β and IL-6 levels after the addition of the remaining tested phenolic acids.

Discussion

In the present study, we observed that dihydroxylated phenolic acids derived from microbial metabolism presented marked *in vitro* anti-inflammatory properties, reducing the secretion of TNF- α , IL-1 β and IL-6 in LPS-stimulated PBMC from healthy subjects. Six different phenolic acids (3,4-DHPPA, 3-HPPA, 3,4-DHPAA, 3-HPAA, 4-HBA and 4-HHA) derived from the microbial metabolism of polyphenols, in particular from monomeric flavanols and proanthocyanidins, were tested at a concentration level (1 μM) within the range (0.1–10 μM) found in plasma samples after the intake of a polyphenol-rich meal and recommended for *in vitro* studies⁽³⁾. Tested compounds were chosen on the basis of their structural features (i.e. hydroxylation pattern and side-chain length of the functional group) and on their abundance in biological fluids after the ingestion of polyphenol-rich foods. To our knowledge, the effects of microbial-derived phenolic acids on the production and release of pro-inflammatory cytokines from human PBMC have not been published previously.

Previous studies based on the consumption of catechin by human subjects resulted in an increase in 3-HPPA⁽¹⁷⁾. Consumption of catechins and proanthocyanidins from chocolate by human subjects resulted in an increased urinary excretion

of 3-HPPA, 3,4-DHPAA, 3-HPAA and 3-HBA⁽¹⁸⁾. More recently, Ward *et al.*⁽¹⁹⁾ described that 3-HPPA was the main metabolite in urine when the human subjects were supplemented with grape-seed polyphenols. Studies on laboratory animals have shown similar results. Main urinary metabolites formed from rats fed a catechin diet were 3-HPPA, 3-HBA and 3-HPA⁽²⁰⁾. Besides these metabolites, 3-HPAA and 4-HBA were detected in the urine of rats fed wine polyphenols⁽²⁰⁾. *In vitro* experiments also confirmed that 3-HPPA was the most abundant metabolite produced from proanthocyanidin polymers⁽⁴⁾ by human colonic microflora, whereas 3,4-DHPAA was the main metabolite from rutin, which was further dehydroxylated to 3-HPAA⁽²¹⁾. This compound was also found to be the major end product of the colonic metabolism of chlorogenic acid by human faecal microbiota *in vitro*⁽²²⁾. The yield of microbial metabolites could be high, in particular for the polyphenols that are poorly absorbed in the small intestine. For example, for chlorogenic and caffeic acids, it represented 57.4 and 28.1% of the total intake, respectively⁽²²⁾. However, in order for these metabolites to be effective at a physiological level, they need to be absorbed and reach target tissues. In fact, once produced by the microbiota, some colonic metabolites could be further absorbed and reach the liver and the kidney where they could be methylated, hydroxylated or conjugated with glycine^(20,23). Recently, the absorption mechanism of some of these colonic metabolites is beginning to be elucidated. Metabolites such as ferulic, *p*-coumaric, *m*-coumaric and 3-HPPA are absorbed by the

monocarboxylic acid transporter, whereas caffeic acid and 3,4-DHPPA permeate across Caco-2 cells via the paracellular pathway^(24–26). Using immunohistochemical tests, Kawai *et al.*⁽²⁷⁾ have recently confirmed that polyphenol metabolites could penetrate the tissues. Quercetin-3-glucuronide, a major metabolite of quercetin, was permeable in LPS-stimulated macrophages, and was converted into the more active aglycone, a part of which was further converted into the methylated form. These data suggest that microbial phenolic metabolites could also undergo a similar pathway in injured cells.

Atherosclerosis is now considered as an inflammatory disease⁽²⁸⁾. Recent epidemiological and clinical studies have shown that the Mediterranean diet or its main components, rich in polyphenols, are associated with a lower inflammatory status⁽²⁹⁾. However, in epidemiological and even in clinical studies, it is difficult to control the effects of the diet consumed and physical activity performed⁽³⁰⁾. Thus, *in vitro* studies allow us to obtain additional information in relation to the direct effect of some compounds (i.e. polyphenol metabolites) in biochemical pathways related to cardiovascular health, such as the production of pro-inflammatory cytokines that participate in the first stages of atherosclerosis. LPS is a bacterial protein and is used as a method to challenge immune cells to produce cytokines, including the inflammatory cytokines. Some of the inflammatory cytokines that are produced are those that have been associated with chronic inflammation and atherosclerosis risk.

The results found in the present study indicate that the effects of the tested phenolic acids on cytokine secretion by PBMC were structure dependent. With the exception of the effects of 4-HHA on TNF- α secretion, only the dihydroxylated phenolic acids, 3,4-DHPPA and 3,4-DHPAA, caused a statistically significant decrease in the secreted levels of the three different cytokines from LPS-stimulated PBMC (Table 1). The standard deviation of the data reflects large inter-individual difference in cytokine secretion among the volunteers. In addition, the degree of inhibition was found to be influenced by the cytokine family. The inhibition of IL-1 β by both compounds was slightly higher than that for TNF- α and IL-6 (Table 1). Monohydroxylated phenolic acids (3-HPPA, 3-HPAA, 4-HBA and 4-HHA) did not produce significant changes in cytokine secretion with the exception of 4-HHA on TNF- α secretion, which produced a significant increase in this cytokine.

The present results on TNF- α , IL-1 β and IL-6 secretion are in agreement with other studies performed with other polyphenols and cell types. Quercetin inhibited the expression of IL-8 and MCP-1 in TNF- α -stimulated synovial cells⁽³¹⁾. Small oligomeric procyanidin fractions (monomer to tetramer) isolated from cocoa reduced the secretion of IL-1 from PHA-stimulated PBMC, whereas polymers (pentamer to decamer) produced an increase in the secreted levels⁽¹²⁾. However, the same fractions promoted the secretion of TNF- α ⁽¹³⁾. Dimeric flavanols isolated from pine bark also enhanced TNF- α levels in stimulated macrophages, while monomers strongly inhibited its secretion⁽³²⁾. Ramiro *et al.*⁽³³⁾ found that epicatechin, isoquercitrin and cocoa extracts decrease the secretion of TNF- α by macrophages in a dose-dependent manner. Also in this line, it has also been reported that several flavones and flavanols inhibited TNF- α secretion by LPS-stimulated macrophages⁽³⁴⁾.

The superior effect of dihydroxylated phenolic acids in comparison with the monohydroxylated ones on the inhibition of pro-inflammatory cytokines has also been reported for other tested biological properties. Thus, 3,4-DHPAA showed more potent cytotoxicity against tumour cell lines than 4-hydroxyphenylacetic acid⁽⁸⁾. 3,4-DHPPA was also among the phenolic acids inhibiting the expression of P-selectin in resting platelets⁽⁵⁾. According to Russell *et al.*⁽³⁵⁾, dihydroxylated phenolic acids present a better antioxidant capacity than monohydroxylated ones due to their stabilisation into quinones.

The mechanism associated with the inhibitory or stimulatory activities of polyphenols on cytokine production may result from transcriptional and post-transcriptional events⁽¹⁴⁾. In fact, NF- κ B, a transcription factor responsible for the activation of a series of cytokines, including TNF- α and IL-1 β , is redox sensitive, and it is well known that the antioxidants such as flavonoids can inhibit its activation⁽³²⁾. Other authors have recently suggested that, besides their antioxidant effects, polyphenols could also function as signalling molecules⁽³⁶⁾.

In this sense, Tedgui & Mallat⁽¹⁰⁾ have suggested that future therapeutic approaches to treat atherosclerosis may include agents that block pro-inflammatory cytokine signalling or the transcription of inflammatory-mediating molecules, among others. The results found in the present study demonstrate that due to their down-regulating effect on the production of pro-inflammatory cytokines TNF- α , IL-1 β and IL-6, polyphenols such as dihydroxylated phenolic acids derived from microbial metabolism could be among the new generation of therapeutic agents for the management of immunoinflammatory diseases such as atherosclerosis.

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