RESEARCH

Enterotoxic potential of dietary cholesterol autoxidation products

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Abstract

Background: In both Western and Westernized diets, together with a relatively high amount of cholesterol, variable amountsof its oxidized metabolites, oxysterols, are consistently consumed. These oxysterols, mostly of non-enzymatic origin, are produced during sterol autoxidation in foodstuff manufacturing and storage.
Objective: This study aimed to analyze the potential enterotoxic effects of all main oxysterols of non-enzymatic origin so far identified in a variety of foods.
Experimental plan: Differentiated human intestinal cell monolayers (CaCo-2) were incubated up to 48 h in the presence or absence of 0.5, 1 or 5 µM with one out of seven non-enzymatic oxysterols, prior to the verification of minimal irreversible cell damage within the chosen concentration range.
Results: All tested oxysterols were proven to exert damaging effects on cell monolayers *in vitro*. The inflammatory interleukin-8 and monocyte chemotactic protein-1 were mostly upregulated by 7-ketocholesterol and 7β-hydroxycholesterol, respectively, then to a lower extent by 5α,6α-epoxycholesterol, 7α-hydroxycholesterol and 5β,6β-epoxycholesterol. 7-Ketocholesterol and 7β-hydroxycholesterol also appeared to be most effective in impairing claudin-1, occludin and E-cadherin proteins, followed by 25-hydroxycholesterol and triol.
Conclusions: The oxysterols consistently derived by food autoxidation were tested; they potentially impaired the integrity of the intestinal epithelial barrier and triggered an inflammatory response within 0.5–5 µM concentrations, easily reachable in a single Western meal.

Significance Statement

This comprehensive analysis focused on the potential impairment of the intestinal barrier by the main dietary non-enzymatic oxysterols, should guide further nutrition research aiming at defining a threshold amount of these cholesterol derivatives in order not to derange the physiological gut–brain axis.

Introduction

Nowadays, a strict functional and bidirectional interconnection between gut and brain is fully recognized, with intestinal microbiota and dietary habits playing a pivotal role in maintaining a physiological equilibrium within such a complex and multifactorial crosstalk (Mayer *et al.* 2015, Cryan *et al.* 2019, Custers *et al.* 2022).

The so-called microbiota–gut–brain axis consists of a complex bidirectional signaling network between the gastrointestinal tract and the brain, where signals deriving from microbiota have a fundamental role not only for ensuring gastrointestinal homeostasis but also for influencing various brain functions. The alteration in the microbiota composition is implied in both the development of intestinal and neuroinflammatory injury (Rutsch *et al.* 2020). The dietary habit that is unanimously considered a potential inducer of marked changes in the function of this axis is implying the uptake of excess amounts of saturated fat and ω6-polyunsaturated fatty acids (Miao *et al.* 2020, Custers *et al.* 2022), typical of Western and Westernized diets. These dietary regimens are indeed characterized by relatively high consumption of fat of animal origin. But the fat of animal source is also rich in cholesterol, and it contains variable amounts of cholesterol oxidation products, deriving either from its enzymatic metabolism or from its autoxidation, the latter reaction occurs mainly during manufacturing processes and storage of foodstuffs (Garcia-Llatas *et al.* 2021, Poli *et al.* 2022a,b).

The oxysterols of enzymatic origin that are constantly present in human tissues and detectable in the nM range in the peripheral blood of healthy subjects are 27-hydroxycholesterol (27OHC), 24-hydroxycholesterol (24OHC), 25-hydroxycholesterol (25OHC) and 7α-hydroxycholesterol (7αOHC) (Dzeletovic *et al.* 1995, Pataj *et al.* 2016). They are recognized to play important physiological functions (Luu *et al.* 2016, Sottero *et al.* 2019), as long as they do not accumulate in disproportionate amounts, in that case contributing to the progression of a variety of non-communicable human diseases (Poli *et al.* 2013, Garcia-Llatas *et al.* 2021, Poli *et al.* 2022a).

Notably, 25OHC and 7αOHC may also be generated by cholesterol autoxidation. This is essentially the case of most of 25OHC and 7αOHC recovered in food ingredients and food products, where no enzymes able to generate them are actually present. There are other oxysterols unequally generated by autoxidation and consistently recovered in a variety of cholesterol-rich foods and then in the gastrointestinal tract, namely, 7-ketocholesterol (7KC), 7β-hydroxycholesterol (7βOHC), 5α,6αepoxide (α-epox), 5β,6β-epoxide (β-epox) and cholestan-3β,5α,6β-triol (triol). All these cholesterol oxides are detectable within the nM range in human peripheral blood (Dzeletovic *et al.* 1995, Pataj *et al.* 2016), meaning that they can be easily absorbed at the gut level, actually following the same absorption pathway of cholesterol (Garcia-Llatas *et al.* 2021, Poli *et al.* 2022b).

All seven 'non-enzymatic' oxysterols reported above have been repeatedly recovered in various foods (Hur *et al.* 2007, Cardenia *et al.* 2013, Brzeska *et al.* 2016, Garcia-Llatas *et al.* 2021, Risso *et al.* 2021) but most likely never quantified all together. It appears difficult to attribute a physiological role to non-enzymatic oxysterols, even if this cannot be excluded, first, because their concentration in the single tissues and organs is highly variable, mostly enhanced by inflammatory processes and not adjustable at all, and secondly, because some of them, in particular, 7KC and 7βOHC have been proven to exert various toxicological effects besides a strong pro-inflammatory action, as very recently reviewed in a comprehensive way (Vejux *et al.* 2020, Nury *et al.* 2021).

The potential toxicity of non-enzymatic oxysterols is now becoming of multidisciplinary interest, not only in human medicine as regards their likely involvement in the progression of several age-related diseases (Poli *et al.* 2013, Zarrouk *et al.* 2014) but also in veterinary medicine, in lanolin-containing cosmetics industry and, last but not least, in the food industry (Canzoneri *et al.* 2022, Poli *et al.* 2022a). Being increasingly recognized by the crucial, physiological role played by the gut–brain functional axis, preventing an excess accumulation of dietary oxysterols turns up as a necessary action to guarantee the quality and safety of foodstuffs containing ingredients of animal origin.

However, while dietary oxysterols come out as harmful compounds for human health, no toxicity threshold for their uptake with food has been defined yet, and thus, comprehensive and quantitative analyses of their possible impact on the epithelial intestinal layer becomes urgent, to orientate toward a specific limitation of their consumption. Besides their cytolytic and pro-inflammatory features, the action of non-enzymatic oxysterols on the selective permeability of intestinal barrier deserves to be investigated in deep. A recent study from our group showed a delocalization and a reduced amount of the tight junctions zonulin-a, occludin and junction-adhesion molecule-A in differentiated CaCo-2 monolayers challenged with a mixture of 7KC, 7αOHC, 7βOHC, 5α-6α-

epox and 5β,6β-epox, at the final concentration of 60 μM (Deiana *et al.* 2017).

Here, we report at the same time on the cell irreversible damage, pro-inflammatory effect and reduced level of intestinal tight and adherens junctions as exerted by all main dietary oxysterols of non-enzymatic origin, added as single compounds to differentiated CaCo-2 cell monolayers, in the final concentrations of 0.5, 1 and 5 μM, amounts that are easily detectable in a large variety of cholesterol-containing foodstuffs.

Materials and methods

Reagents

All reagents and chemicals were obtained from Sigma Aldrich Srl, unless otherwise specified. DMEM with high glucose content, fetal bovine serum (FBS) and trypsin (5 g/L solution) were from S.I.A.L. Srl (Rome, Italy). Multiwell plates and tissue culture flask were from VWR International Srl (Milan, Italy). Milli-Q filter system ultrapure water was from Millipore. Protease inhibitor cocktail 'cOmplete ULTRA Tablets Mini EASYpacks' was obtained from Roche SpA. Bio-Rad protein assay dye reagent and ECL®Western Blotting System were from Bio-Rad Srl. Hybond ECL nitrocellulose membrane was from GE Healthcare Srl. The 4× lithium dodecyl sulfate (LDS) solution and 10× dithiothreitol (DTT) reducing agent were supplied by Thermo Fisher Scientific (Life Technologies Italia). Mouse anti-claudin-1 (SC- 166338) polyclonal primary antibody, mouse anti-E-cadherin (14472S) and rabbit anti-occludin (91131S) monoclonal primary antibodies, anti-mouse IgG and anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (7076S and 7074S, respectively) were supplied by Cell Signaling Technology. The kits interleukin-8 (IL-8) (DY208) and monocyte chemoattractant protein-1 (MCP-1) (DY279) detection by ELISA were from R&D System. Oxysterols α-epox, β-epox, 7KC, 7αOHC and 7βOHC were from Avanti Polar Lipids (Alabaster, AL, USA); 25OHC and triol were obtained from Sigma Aldrich Srl.

Cell culture and treatments

Human colorectal adenocarcinoma CaCo-2 cells were supplied by the Cell Bank Interlab Cell Line Collection (Genoa, Italy). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 1% antibiotic/antimycotic solution (containing 100 U/mL penicillin, 0.1 mg/mL streptomycin, 250 ng/mL amphotericin B and 0.04 mg/mL gentamicin) at 37°C in a humidified atmosphere containing 5% CO_2. For cellular treatments, CaCo-2 cells were plated at 1×10^6/mL density; after reaching confluence, cells were cultured for further 18 days. Upon reaching confluence, this human epithelial cell line starts a spontaneous differentiation process into enterocyte-like phenotype. The differentiated CaCo-2 cell monolayer expresses morphological and biochemical properties typical of mature small intestinal enterocytes showing a typical brush border with microvilli at the apical side and with tight junction complexes between adjacent cells. Differentiated CaCo-2 cells were incubated overnight in serum-free medium to make them quiescent. Cell medium was then replaced with DMEM containing 1% FBS and differentiated CaCo-2 cells were treated with different concentrations (0.5, 1, 5, 10, 20, 50 and 100 μM) of 7KC, 7βOHC, 7αOHC, β-epox, α-epox, 25OHC and triol for 15, 24 and 48 h.

Cell death evaluation

Differentiated CaCo-2 cells were treated with increasing concentrations of oxysterols at different times (see 'Cell culture and treatments' and 'Results'). The percentage of lactate dehydrogenase (LDH) cellular release into the culture medium was used as the parameter of cytolysis.

LDH was evaluated spectrophotometrically at 340 nm wavelength by recording NADH production/min. 100% LDH release reference value was obtained by evaluating the enzyme release after the complete cell lysis obtained by adding 0.5% Triton X-100 to the plate containing the same cell density as treated cells. Untreated cells were considered as control.

Immunoblotting

At the end of each treatment, differentiated CaCo-2 cells were washed with ice-cold PBS 1× and scraped. For protein extraction, 150 μL of lysis buffer (PBS 1× supplemented with 1% Triton X-100 (v/v), 1% sodium dodecyl sulfate (w/v) (final volume)) was added to each sample. Lysates were incubated for 30 min on ice and centrifugated at 12,052 g at 4°C for 15 min. Bio-Rad protein assay dye reagent was used for the evaluation of total cell extract protein concentration. Samples, containing 50 μg total

proteins, were boiled at 100°C for 5 min in the sample buffer (LDS sample buffer 4× and DTT sample reducer 10×). Boiled samples were subjected to electrophoresis separation, and subsequently, proteins were transferred to the Hybond ECL nitrocellulose membranes. Saturation of nonspecific binding sites was performed at room temperature (RT) for 1 h with TTTBS blocking buffer (TTBS: tris-buffered saline (TBS) supplemented with 0.05% (v/v) Tween 20) plus 5% (w/v) skimmed milk powder (final volume). Then, the membranes were incubated at 4°C overnight with mouse anti-claudin-1 (1:500) polyclonal primary antibody, rabbit anti-occludin (1:800 dilution) and mouse anti-E-cadherin (1:800 dilution) monoclonal primary antibodies in TBS containing 0.1% Tween-20 (v/v) and 3% BSA (w/v). Three washes in TTBS were done and blots were incubated with anti-mouse IgG or anti-rabbit IgG HRP-conjugated secondary antibody (1:1000 dilutions) in TBS with 0.1% Tween-20 (v/v) and 3% BSA (w/v) for 1 h at RT. Blots were then washed twice in TTBS for 10 min. Chemiluminescence was detected using the kit Clarity Western ECL and the ChemiDoc™ Touch Imaging System machine (Bio-Rad laboratories Srl). ImageJ Software (Bethesda, MD, USA) was used to quantify protein band densities.

Enzyme-linked immunoassays (ELISA)

The extracellular protein levels of IL-8 and MCP-1 were quantified in the collected cell culture medium by using commercial ELISA kits according to the manufacturer's instructions. Sample absorbance values were read in a 96-multiwell plate reader (Model 680 Microplate Reader, Bio-Rad laboratories Srl) at 450 nm wavelength; the optical densities recorded at 655 nm were used as reference value. Cytokine levels were normalized for total proteins present in the corresponding culture medium volume. Total protein concentration was evaluated in each sample by using Bio-Rad protein assay dye. The analyses were performed in triplicate and values were expressed as pg cytokines/mg cell culture medium proteins.

Statistical analysis

Results were expressed as mean ± S.D. and data were analyzed with GraphPad InStat software. The statistical differences among experimental data were evaluated by using the one-way ANOVA test associated with Bonferroni's multiple comparison post-test.

Results

Oxysterol-induced irreversible damage measured in terms of extracellular release of lactate dehydrogenase

To evaluate the potential cytotoxic effect of the oxysterols of full (7KC, 7βOHC, α-epox, β-epox, triol) or partial (7αOHC, 25OHC) non-enzymatic origin, actually detectable in biological fluids, differentiated CaCo-2 cell cultures were challenged with seven different concentrations (0.5, 1, 5, 10, 20, 50 and 100 μM) of each considered oxysterol, at three different incubation times (15, 24 and 48 h), and the results are summarized in Table 1.

The differentiated CaCo-2 cells were chosen because they are well recognized as an *in vitro* model of intestinal epithelial barrier. CaCo-2 capacity to spontaneously differentiate into a monolayer of cells showing distinct features of absorbent enterocytes makes them a perfect experimental system for studying the mechanisms of intestinal metabolism and transport. Meanwhile, this culture model provides important details on absorption, distribution, metabolism and excretion of drugs and dietary components (Lea 2015).

All tested oxysterols showed a progressive increase in extracellular LDH release from the cell layers incubated for 15 h up to 48 h. Such an enzyme release resulted to be statistically significant at 48 h for all oxysterols except one, namely, the triol, already at 1 μM final concentration. Still, at 48 h cell incubation time, 7KC, 25OHC and 7αOHC exhibited significant cytotoxicity also at 0.5 μM final concentration. Moreover, the cytotoxicity exerted by 7KC was already significantly evident at 15 and 24 h incubation times, while the oxysterol inducing the lowest cytotoxic effect was the triol (Table 1).

When comparing the time- and concentration-dependent cytotoxic potential of the seven oxysterols examined, 7βOH, α-epox and β-epox appeared to be the most harmful compounds, followed by 7KC, then by 7αOHC and 25OHC, the triol confirming to be the less toxic cholesterol oxide (Fig. 1).

Oxysterol-induced inflammatory response by intestinal epithelial cell line CaCo-2

With the aim to assay if an inflammatory response was triggered in differentiated CaCo-2 cell cultures by the treatment with the various non-enzymatic oxysterols, cells were incubated for 24 h in the presence of 0.5, 1 or 5 μM oxysterols' concentrations, namely, a condition

Table 1 Oxysterol-dependent cytolysis in differentiated CaCo-2 cells.

	Control	0.5 µM	1 µM	5 µM	10 µM	20 µM	50 µM	100 µM
15 h								
7KC	1.14 ± 0.14	2.90 ± 0.42*	3.16 ± 0.71*	3.33 ± 0.44**	3.35 ± 0.57**	3.48 ± 0.59**	6.03 ± 0.26***	9.55 ± 0.13***
7βOHC	1.12 ± 0.03	1.70 ± 0.78	2.50 ± 0.64*	3.29 ± 0.52**	4.08 ± 0.25**	6.35 ± 0.89***	9.62 ± 0.41***	11.72 ± 0.51***
7αOHC	0.93 ± 0.08	1.41 ± 0.29	1.93 ± 0.47	2.66 ± 0.69*	3.69 ± 0.90**	4.55 ± 0.54***	6.16 ± 0.28***	8.35 ± 0.58***
β-epox	1.21 ± 0.00	1.83 ± 0.12	2.24 ± 0.20	2.85 ± 0.13*	4.32 ± 0.14**	5.93 ± 0.27***	9.16 ± 1.75***	10.65 ± 0.61***
α-epox	1.03 ± 0.01	1.55 ± 0.43	2.48 ± 0.43*	3.24 ± 0.25**	4.34 ± 0.22***	5.82 ± 0.31***	8.82 ± 0.42***	10.29 ± 0.77***
25OHC	1.01 ± 0.03	1.69 ± 0.06	2.12 ± 0.06	2.81 ± 0.31**	3.57 ± 0.32***	5.33 ± 0.37***	6.21 ± 0.24***	8.25 ± 0.65***
triol	1.23 ± 0.01	1.62 ± 0.10	2.18 ± 0.11	2.83 ± 0.28*	3.42 ± 0.05***	5.37 ± 0.21***	6.58 ± 0.70***	7.39 ± 0.85***
24 h								
7KC	1.46 ± 0.05	3.84 ± 0.70*	4.06 ± 0.78*	4.34 ± 0.60**	4.78 ± 0.49**	5.24 ± 0.09***	7.12 ± 0.049***	11.84 ± 1.06***
7βOHC	1.48 ± 0.04	2.05 ± 0.65	3.57 ± 0.18*	4.49 ± 0.44*	6.58 ± 0.79***	7.99 ± 1.35***	11.14 ± 1.06***	14.49 ± 1.34***
7αOHC	1.29 ± 0.27	1.40 ± 0.15	1.90 ± 0.31	3.78 ± 0.09***	5.03 ± 0.25***	6.43 ± 0.22***	7.17 ± 0.14***	10.35 ± 0.52***
β-epox	1.59 ± 0.32	2.45 ± 0.28	3.73 ± 0.21**	4.90 ± 0.20***	6.48 ± 0.47***	7.99 ± 0.44***	8.62 ± 0.46***	13.07 ± 0.32***
α-epox	1.30 ± 0.30	2.09 ± 0.27	3.12 ± 0.13*	5.72 ± 1.03***	6.63 ± 0.35***	7.35 ± 0.12***	10.01 ± 0.59***	12.96 ± 0.39***
25OHC	1.32 ± 0.29	1.73 ± 0.06	2.22 ± 0.12	2.86 ± 0.15*	5.44 ± 0.37***	7.65 ± 0.21***	8.49 ± 0.43***	10.41 ± 0.18***
triol	1.54 ± 0.25	1.80 ± 0.04	2.43 ± 0.26	2.66 ± 0.19	4.90 ± 0.46***	6.69 ± 0.54***	6.82 ± 0.37***	7.40 ± 0.50***
48 h								
7KC	1.86 ± 0.04	5.01 ± 1.28*	5.84 ± 1.32**	5.83 ± 1.01**	6.52 ± 0.55***	7.00 ± 0.45***	9.96 ± 0.99***	15.05 ± 1.04****
7βOHC	1.67 ± 0.01	2.56 ± 0.30	4.14 ± 0.78*	6.40 ± 1.85**	8.19 ± 1.13***	9.57 ± 0.36***	11.82 ± 0.60***	18.61 ± 0.54****
7αOHC	1.50 ± 0.25	2.47 ± 0.74*	3.73 ± 1.02***	5.18 ± 0.76***	6.36 ± 0.85***	1.56 ± 0.49***	9.62 ± 0.31***	11.79 ± 0.72****
β-epox	1.57 ± 0.02	2.49 ± 0.15	4.14 ± 0.82***	6.56 ± 0.42***	8.21 ± 0.19***	9.31 ± 0.15***	11.20 ± 0.30***	17.99 ± 0.66****
α-epox	1.50 ± 0.33	2.62 ± 0.09	3.77 ± 0.27**	6.45 ± 0.58***	8.35 ± 0.32***	10.13 ± 0.34***	11.52 ± 0.34***	17.86 ± 0.47****
25OHC	1.53 ± 0.17	3.04 ± 0.11**	4.80 ± 0.14***	5.27 ± 0.28***	6.71 ± 0.25***	8.02 ± 0.12***	10.12 ± 0.57***	11.60 ± 0.30***
triol	1.68 ± 0.02	1.88 ± 0.09	2.77 ± 0.21	3.62 ± 0.24**	6.02 ± 0.41***	8.32 ± 0.09***	8.44 ± 0.65***	10.42 ± 0.21***

Cell damage was evaluated as percentage of LDH released in the culture media due to cell treatment with different concentrations (0.5, 1, 5, 10, 20, 50 and 100 µM) of single oxysterols for 15, 24 and 48 h. The percentage was evaluated as described in the 'Materials and methods' section. Data are reported as means ± s.d. of three independent experiments. Statistical analyses were performed by using ANOVA with Bonferroni post-test. Significantly different vs control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

previously checked to exert a minimal cytotoxicity (Table 1); then, the cellular excretion of the inflammatory cytokines IL-8 and MCP-1 into the culture medium was quantified by the ELISA method.

The cell excretion of the neutrophil chemotactic factor IL-8 appeared to be significantly induced at the 5 µM concentration by all oxysterols tested except one, namely, the triol (Fig. 2). The most efficient pro-inflammatory stimulus was provided by 7KC, followed by 7βOHC and α-epox. Moreover, 7KC was inducing a significant cell excretion of IL-8 at 1 µM concentration and 7αOHC to a minor extent (Fig. 2).

All tested oxysterols significantly stimulated the CaCo-2 cell excretion of the MCP-1 when added to the cell cultures at the 5 µM concentration (Fig. 3). 7βOHC and α-epox, but not 7KC, showed a significant enhancement of this chemokine's excretion at 1 µM concentration. Somehow unexpectedly, the triol also exhibited a significant while low enhancement of MCP-1 excretion at 1 µM concentration (Fig. 3).
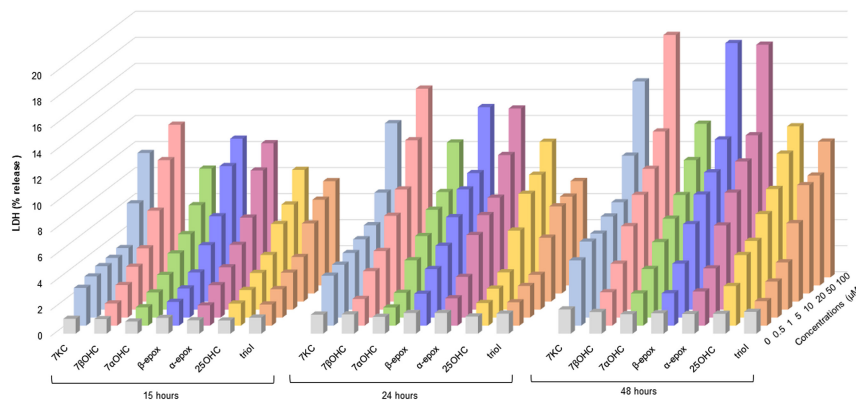

Figure 1
LDH release was evaluated in the culture media of differentiated CaCo-2 cells treated with different concentrations (0.5, 1, 5, 10, 20, 50 and 100 µM) of 7KC, 7βOHC, 7αOHC, β-epox, α-epox and 25OHC and triol for 15, 24 and 48 h. LDH was estimated as percentage referred to 100% cell enzyme released into the medium following the addition of 0.5% Triton X-100 to the cultured cells grown at the same density as other samples. Data are reported as means of three independent experiments.
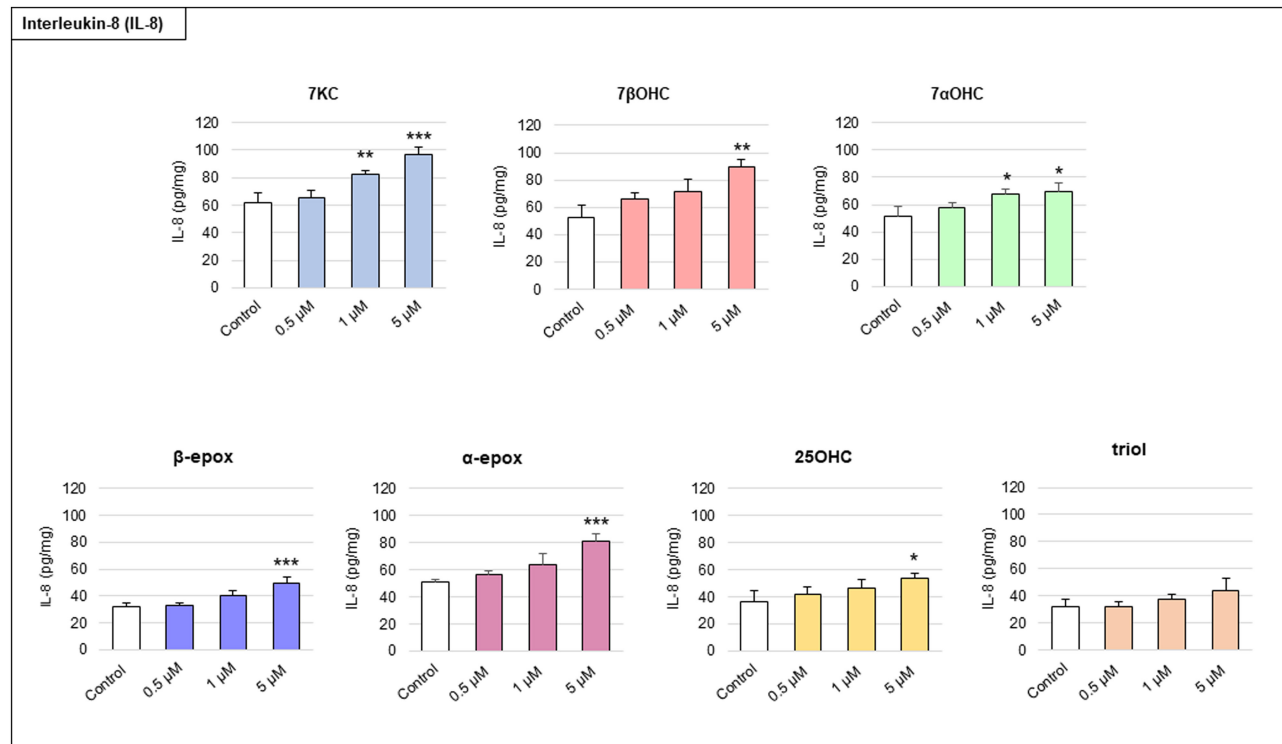
Figure 2

Differentiated CaCo-2 cells were incubated with three different concentrations (0.5, 1 and 5 µM) of 7KC, 7βOHC, 7αOHC, β-epox, α-epox, 25OHC and triol for 24 h. IL-8 protein levels were quantified by ELISA in the cell culture medium. Control: untreated cells. Values are shown as pg chemokines normalized for cell incubation medium mg proteins and are expressed as means ± s.d. of three independent experiments. Statistical analyses were performed by using ANOVA with Bonferroni post-test. Significantly different vs control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Reduced synthesis of tight and adherens junctions' proteins in intestinal epithelial CaCo-2 cells challenged with the seven investigated non-enzymatic oxysterols

The evidence of significant cytotoxic and pro-inflammatory effects as exerted by all tested oxysterols, also in the low micromolar concentration range, indirectly suggested as being likely impairment of the semi-permeability of the intestinal epithelial layer, due to a derangement of tight and possibly of adherens junctions. Indeed, such a derangement was previously demonstrated to be provoked by the treatment of differentiated CaCo-2 cell layers with a mixture of non-enzymatic oxysterols but given in one order of magnitude higher amount, namely, 60 µM (Deiana *et al.* 2017).

Regarding the most superficial TJ protein in the intestinal epithelium, namely, claudin-1, all seven oxysterols provoked a reduction of its synthesis from 30 to 50%, when added to differentiated CaCo-2 cells at 5 µM final concentration (Fig. 4), with 7KC being the compound exerting the strongest effect. When the dosing concentration of the oxysterols was reduced to 1 µM,

only 7βOHC, 25OHC and triol maintained a significant inhibitory effect (Fig. 4).

Only three out of seven tested oxysterols showed significant inhibition in the synthesis of the less superficial TJ, namely, occludin, i.e. 7KC and to a lesser extent 7βOHC and 25OHC. The latter oxysterol was showing a significant inhibitory effect down to the 0.5 µM concentration, an inhibition apparently not dose-dependent (Fig. 5).

When measuring the level of E-cadherin in differentiated CaCo-2 cells, 7βOHC and 7KC showed a dose-dependent and strong inhibition of the synthesis of this adherens protein, statistically significant at 1 µM final concentration (Fig. 6). Two other oxysterols, i.e. α-epox and triol, showed some significant while modest inhibitory activity but only when added to the intestinal cell cultures at 5 µM final concentration (Fig. 6).

Discussion

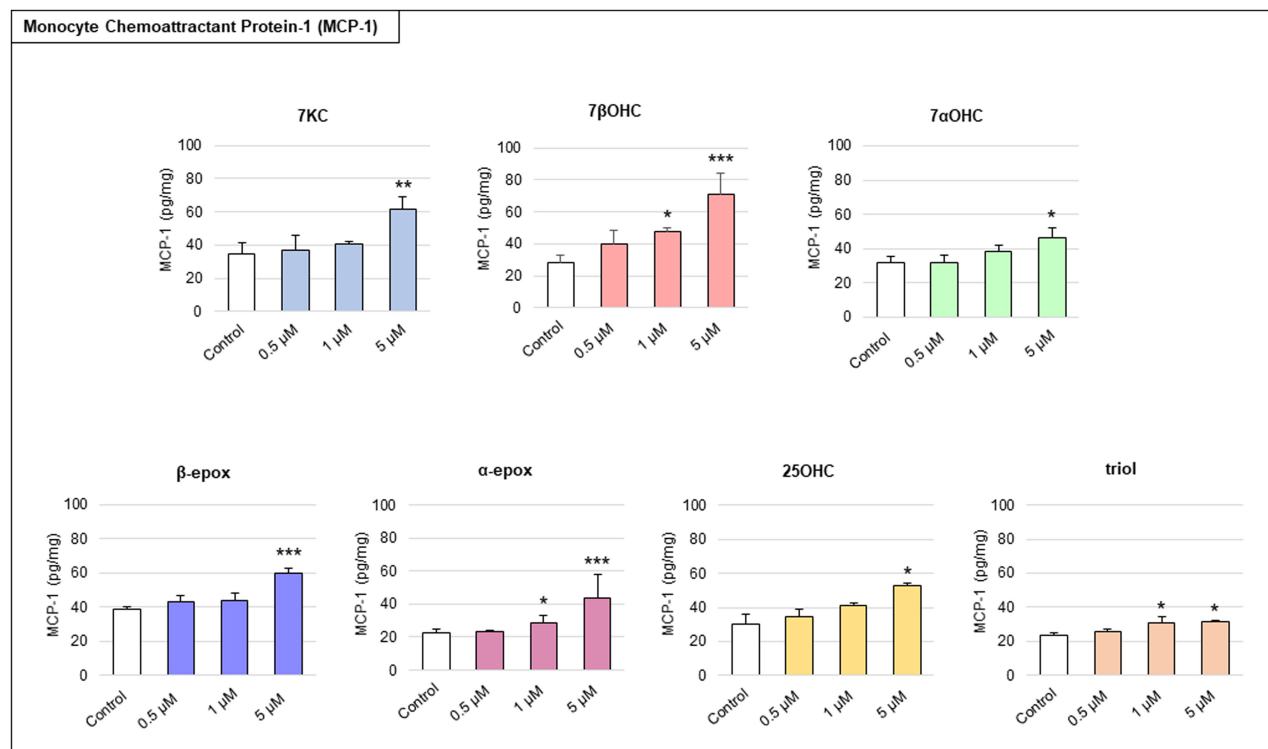All oxysterols tested are of recognized pathophysiological interest, five of uniquely non-enzymatic origin (7KC,

Figure 3
Differentiated CaCo-2 cells were incubated with three different concentrations (0.5, 1 and 5 μM) of 7KC, 7βOHC, 7αOHC, β-epox, α-epox, 25OHC and triol for 24 h. MCP-1 protein levels were quantified by ELISA in the cell culture medium. Control: untreated cells. Values are shown as pg chemokines normalized for cell incubation medium mg proteins and are expressed as means ± s.d. of three independent experiments. Statistical analyses were performed by using ANOVA with Bonferroni post-test. Significantly different vs control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

7βOHC, α-epox, β-epox, triol) and two stemming both from enzymatic reactions and autoxidation (7αOHC, 25OHC). Clearly, 7αOHC and 25OHC detectable in food and cholesterol-rich diets essentially stem from autoxidation reactions.

Indeed, these oxysterols have been identified in a variety of foods (Cardenia *et al.* 2013, Garcia-Llatas *et al.* 2021, Poli *et al.* 2022*a*) and are actually detectable in human peripheral blood in nM amounts (Dzeletovic *et al.* 1995, Pataj *et al.* 2016). All of them have shown, in the reported standard *in vitro* model presented here, to be potentially enterotoxic already in the concentration range of 0.5–5 μM. Of note, most of the previous toxicologic investigations, while thorough and extremely informative, were mainly focused on the two apparently most toxic oxysterols of non-enzymatic origin, namely, 7KC and 7βOHC (Vejux *et al.* 2020, Nury *et al.* 2021). In addition, the concentrations of these two oxysterols employed in *in vitro* studies were often one order of magnitude higher than those employed in the research reported here (see for a review Vejux *et al.* 2020).

A further important feature of the present experimental study was the evaluation of a possible pro-inflammatory response by epithelial intestinal layers (of differentiated CaCo-2 cells) *in vitro* challenged with amounts of single non-enzymatic oxysterols not or poorly inducing acute irreversible cell damage (Table 1), in a way acting like Trojan horses.

This experimental approach allowed us to demonstrate that all the non-enzymatic oxysterols of pathophysiological relevance might induce a net chemotactic reaction by differentiated CaCo-2 cell monolayers, in terms of upregulated synthesis and secretion of the neutrophil chemokine IL-8 and monocyte chemokine MCP-1 (Figs. 2 and 3). 7βOHC, followed by 7KC, confirmed to may exert a robust pro-inflammatory effect, even in the lowest μM range, being most of the challenged intestinal cells still alive and functioning. Interesting was the observation of some significant pro-inflammatory property exhibited, in particular by α-epox, and β-epox the metabolism of which appears different from that of the other
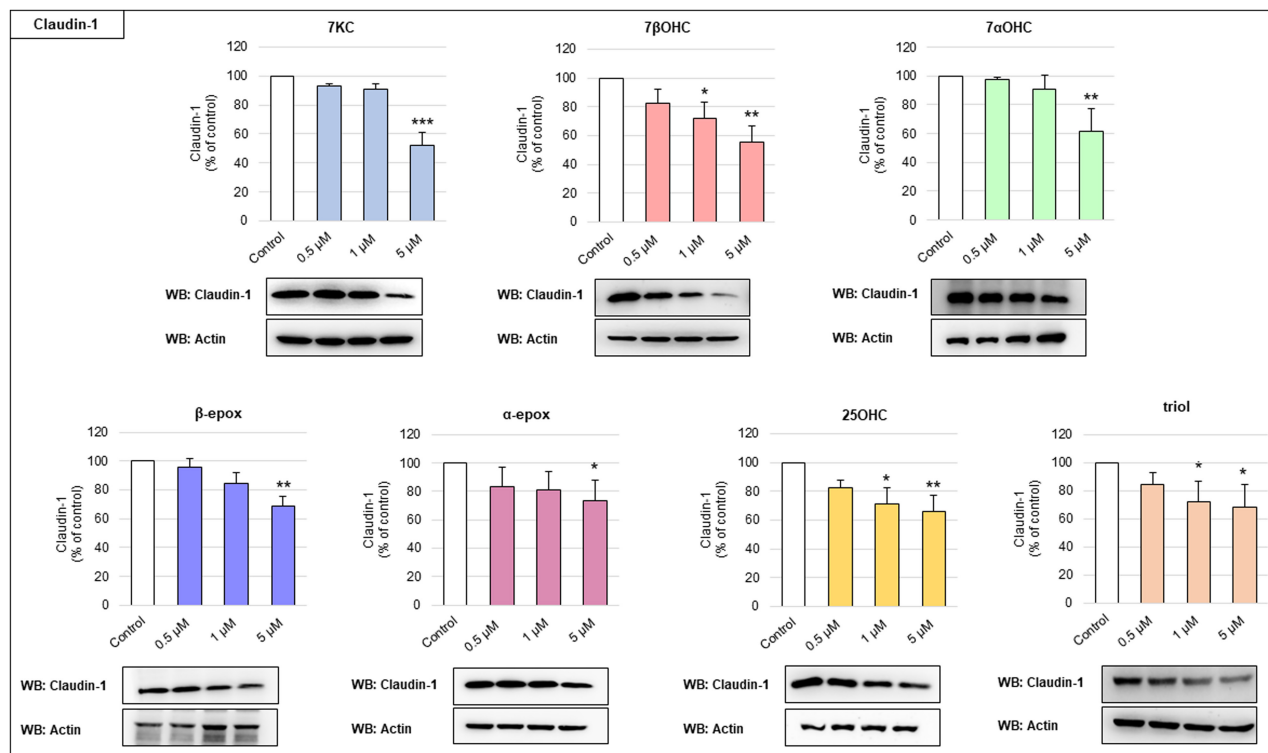
Figure 4

Claudin-1 protein levels were evaluated by Western blotting in the lysates of CaCo-2 cells treated with different concentrations (0.5, 1 and 5 μM) of 7KC, 7βOHC, 7αOHC, β-epox, α-epox, 25OHC and triol for 24 h. Control: untreated cells. Data are expressed as percentage of control (considered as 100%). Values are means $\pm$ s.d. of three independent experiments. Statistical analyses were performed by using ANOVA with Bonferroni post-test. Significantly different vs control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

examined compounds, involving a cholesterol epoxide hydrolase instead of cytochromes 27A1 and 7B1 or a sulfotransferase (Mutemberezi *et al.* 2016).

Previous studies on CaCo-2 cells treated with a mixture of dietary oxysterols showed that these compounds were able to induce a reactive oxygen species (ROS) overproduction by the induction of the NADPH oxidase1 (NOX1). Both ROS and NOX1 were increased in differentiated cells more than in undifferentiated ones (Biasi *et al.* 2009). Subsequent studies, with antioxidant phenolic compound pretreatments of CaCo-2 cell monolayers, confirmed the oxysterol mixture action in inducing oxidative stress and inflammatory cytokine production in differentiated cells primarily involving NOX1, JNK1/2-p38 MAPK and NF-κB signaling axis (Guina *et al.* 2015, Serra *et al.* 2018).

Those studies mainly concerned the combined action of oxysterols, hypothesizing that they are present in dietary lipids as a mixture and this condition could mimic their effects on the intestinal mucosa. But we cannot exclude that the same oxysterols used in mixture in those

experiments can elicit a different response if used singularly. For instance, different responses of single oxysterols used in same concentrations as present in mixture were observed. In particular, the effect of 7βOHC alone appeared more marked than that of the oxysterol mixture in the activation of inflammatory cytokine expression such as IL-8, IL-1α, IL-6, MCP-1 and IL-23 (Mascia *et al.* 2010). Therefore, it is important to understand which oxysterols are more toxic than others.

As for the inflammatory response triggered in living differentiated CaCo-2 cell monolayers, all tested non-enzymatic oxysterols, individually administered, were demonstrated to induce, at the lowest micromolar concentrations, a net impairment of the intercellular junctions by significantly reducing the steady-state levels of the tight junction proteins claudin-1 and occludin (Figs. 4 and 5) and of the adherens junction protein E-cadherin (Fig. 6). Once again, 7βOHC and 7KC appeared as the most efficient non-enzymatic oxysterols in deranging, in a multifocal way, the structure of the epithelial intestinal layers. Of interest as well was the
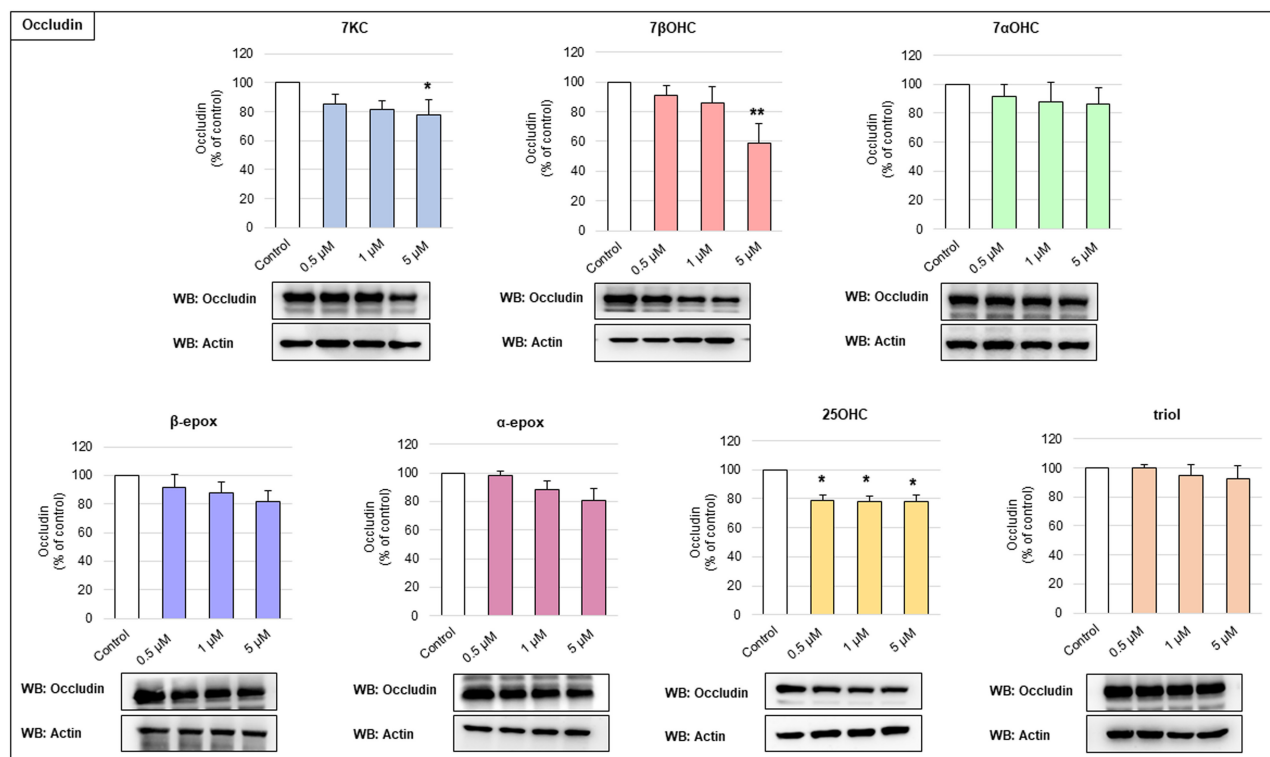
Figure 5
Occludin protein levels were evaluated by Western blotting in the lysates of CaCo-2 cells treated with different concentrations (0.5, 1 and 5 µM) of 7KC, 7βOHC, 7αOHC, β-epox, α-epox, 25OHC and triol for 24 h. Control: untreated cells. Data are expressed as percentage of control (considered as 100%). Values are means ± S.D. of three independent experiments. Statistical analyses were performed by using ANOVA with Bonferroni post-test. Significantly different vs control: *$P < 0.05$, **$P < 0.01$.

effect of 25OHC, in particular, on claudin-1 and occludin protein levels.

The structural damage of the intestinal barrier by dietary oxysterols, as sharply suggested by the *in vitro* findings reported here, might imply a dangerous impairment of the gut–brain functional axis, especially if the damage would become repeatedly induced. The latter condition could take place in individuals consistently fed a diet overly rich in cholesterol. Dietary oxysterols are absorbed at the intestinal level together with cholesterol (Garcia-Llatas *et al.* 2021, Poli *et al.* 2022b) and likely, the most toxic ones could exert on the endothelial layer of the blood–brain barrier pro-inflammatory and intercellular junctions deranging effects like those exerted on the intestinal barrier (Poli *et al.* 2022b). In this way, oxysterols should easily pass the barrier itself, due to being less lipophilic and much more biochemically reactive than the cholesterol parental compound (Gamba *et al.* 2015).

In this relation, impressive was the progressive increase of various oxysterols detected in Alzheimer's disease (AD)

brains in postmortem, classified by the Braak staging system of neurofibrillary pathology, with two compounds already significantly increased in early AD cases, namely, 7βOHC and 7KC (Testa *et al.* 2016). A sustained impairment of the gut–brain axis is increasingly recognized as a promoter of various both psychiatric and neurological disorders (Cryan *et al.* 2019, Custers *et al.* 2022), and hence, some oxysterols at least should be considered among the primary risk factors for these pathologies.

Moreover, at the gut level, chronic damage of the intestinal luminal surface affecting its highly selective permeability would create an abnormal passage toward the sub-epithelial space exploited by the gut microbiota, in particular, opportunistic pathogens (Poli *et al.* 2022b). Thus, one should expect an oxysterol-dependent impairment of the microbiota–gut–brain axis playing a role in brain functional and structural disorders.

In conclusion, besides a low to moderate intake of cholesterol-containing food, it appears a nutritional priority to limit to a minimum the autoxidation of food cholesterol, not only by suitable intake of antioxidants
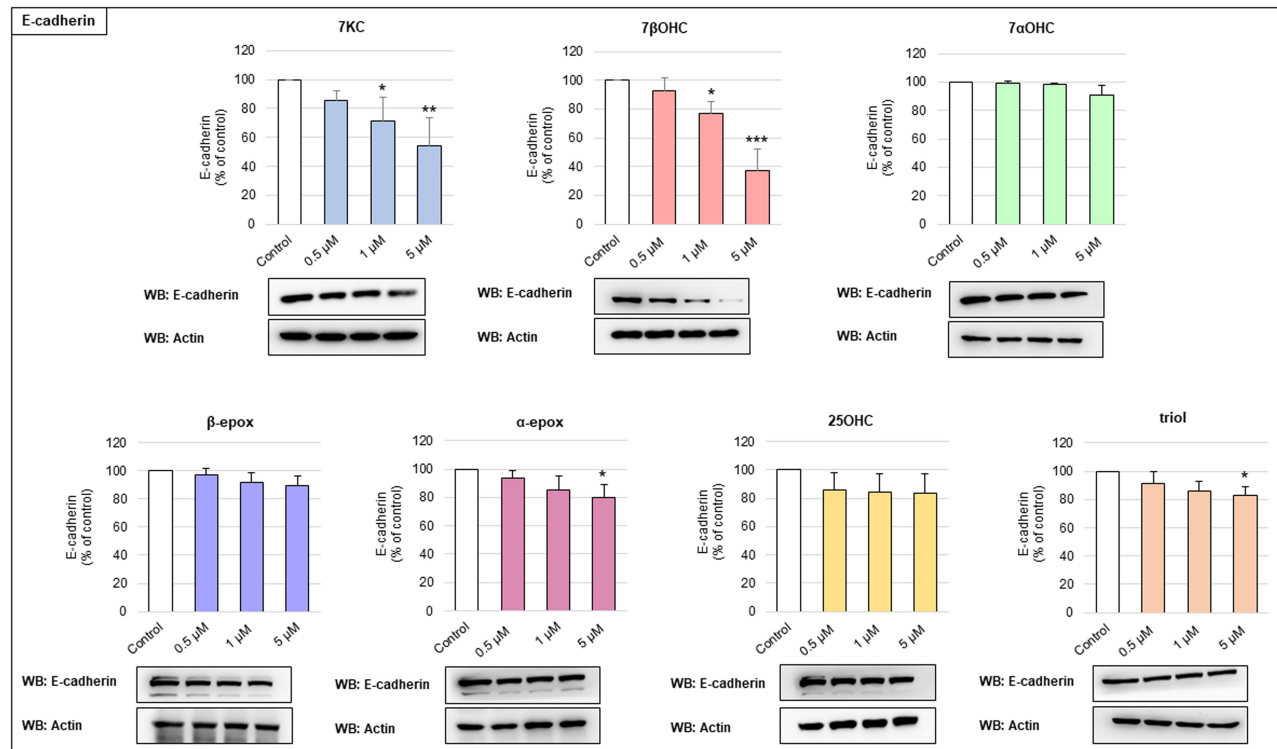
Figure 6

E-cadherin protein levels were evaluated by Western blotting in the lysates of CaCo-2 cells treated with different concentrations (0.5, 1 and 5 μM) of 7KC, 7βOHC, 7αOHC, β-epox, α-epox, 25OHC and triol for 24 h. Control: untreated cells. Data are expressed as percentage of control (considered as 100%). Values are means ± s.d. of three independent experiments. Statistical analyses were performed by using ANOVA with Bonferroni post-test. Significantly different vs control: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

from fruits and vegetables but also by improving the production, the packaging (Canzoneri *et al.* 2022 submitted manuscript), the storage and the cooking of foodstuffs.

Author contribution statement

FB, FC and RM conceived and supervised the overall study; FB and FC wrote the manuscript; NI performed the immunoblotting and enzyme-linked immunoassays with the collaboration of GR.

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