Efficacy of theobromine in preventing intestinal CaCo-2 cell damage induced by oxysterols

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Efficacy of Theobromine cocoa bean shell fraction in preventing intestinal CaCo-2 cell damage induced by oxysterols

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Corresponding Author:	Fiorella Biasi Universita Degli Studi di Torino Dipartimento di Scienze Cliniche e Biologiche Orbassano, Turin ITALY
First Author:	Noemi Iaia
Order of Authors:	Noemi Iaia
	Daniela Rossin
	Barbara Sottero
	Ivana Venezia
	Giuseppe Poli
	Fiorella Biasi
Abstract:	The alteration of the intestinal barrier function is currently believed to be involved in the pathogenesis of gut diseases mainly associated with the activation of inflammation processes. Diet plays an important role in the control of human gut integrity. Theobromine is a natural methylxanthine present in dark chocolate particularly abundant in cocoa bean shell. This is a polyphenol rich by-product generated in cocoa industrial processing, which is gaining value as a functional ingredient. This study aims to highlight for the first time the capability of theobromine in protecting the intestinal cell monolayer from a mixture of dietary oxysterols showing an inflammatory action in terms of IL-8 and MCP-1 overproduction. Differentiated CaCo-2 cells were treated with 60 μM oxysterol mixture and pre-incubated with 10 μM theobromine. Intestinal barrier damage was investigated in terms of tight junction claudin 1, occludin and JAM-A protein levels, matrix metalloproteinase (MMP-2 and MMP-9) activation and anti/pro-apoptotic protein changes. The observed cell monolayer permeability protection by theobromine may be due to its ability to inhibit the production of cytokines and matrix metalloproteinase that can be responsible for tight junction loss and apoptosis in intestinal cells. Our findings provide additional mechanistic hints on the healthy effect of theobromine cocoa component as an attractive natural molecule in the prevention of inflammatory gut diseases.
Suggested Reviewers:	GIUSEPPE VALACCHI gvalacc@ncsu.edu
	Gérard LIZARD Gerard.Lizard@u-bourgogne.fr
	Francesca LUCHETTI francesca.luchetti@uniurb.it
	Irundika DIAS h.k.i.dias1@aston.ac.uk
Opposed Reviewers:	
Order of Authors (with Contributor Roles):	Noemi Iaia (Conceptualization: Equal; Investigation: Equal; Writing – original draft: Equal; Writing – review & editing: Equal)
	Daniela Rossin (Investigation: Equal)
	Barbara Sottero (Validation: Lead)

	Ivana Venezia (Investigation: Supporting; Writing – original draft: Supporting)
	Giuseppe Poli (Supervision: Equal)
	Fiorella Biasi (Conceptualization: Equal; Funding acquisition: Lead; Supervision: Lead; Writing – original draft: Equal; Writing – review & editing: Equal)

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We are submitting for consideration the original article titled "Efficacy of Theobromine cocoa bean shell fraction in preventing intestinal CaCo-2 cell damage induced by oxysterols" (Authors: Iaia N., Rossin D., Sottero B., Venezia I., Poli G. and Biasi F.), to be published in *Archives of Biochemistry and Biophysics*, Special Issue: Polyphenols and Food Factors on Health

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We suggested during online submission four names of experts we deem appropriate as potential referees of the present manuscript.

Looking forward to hearing from you

With best regards

Orbassano, July 2, 2020

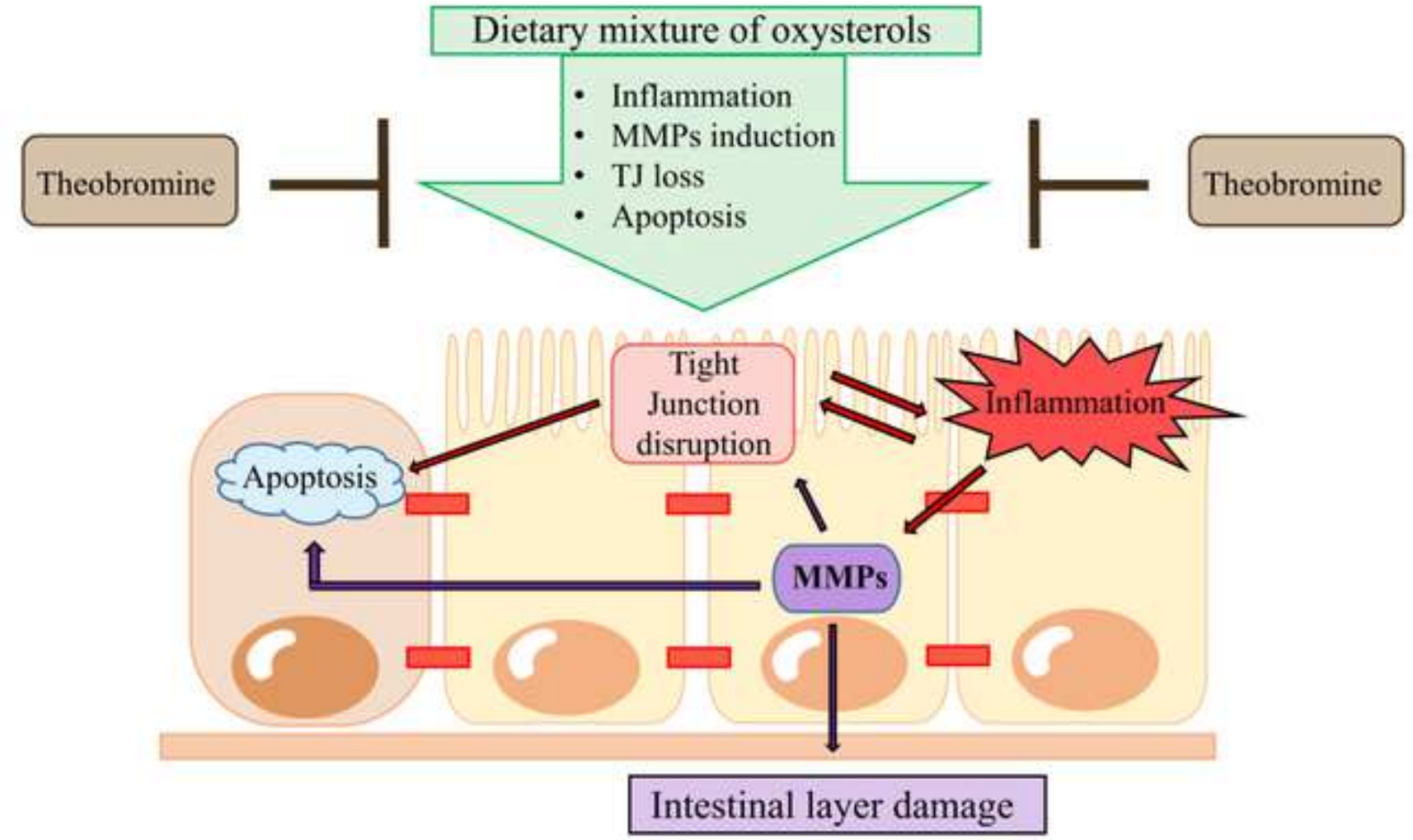
Fiorella Biasi - corresponding author

Department of Clinical and Biological Sciences,
University of Turin at San Luigi Gonzaga Hospital,
Regione Gonzole 10, 10043 Orbassano, Torino, Italy.
Phone: +39 0116705420; Fax: +39 0116705424;
e-mail: fiorella.biasi@unito.it

HIGHLIGHTS

- Theobromine (TB), other than polyphenols, is the main component of cocoa bean shells
- TB prevents intestinal inflammation induced by dietary oxysterols in mixture (Oxy-mix)
- TB prevents TJ loss and MMP activation in CaCo-2 cell monolayer due to Oxy-mix action
- TB inhibits Oxy-mix-induced apoptosis by restoring Bax/Bcl-xL protein level changes
- TB can be partly responsible for the benefits associated with cocoa consumption

Graphical Abstract



Efficacy of Theobromine cocoa bean shell fraction in preventing intestinal CaCo-2 cell damage induced by oxysterols

Noemi Iaia[a], Daniela Rossin[a], Barbara Sottero[a], Ivana Venezia[a], Giuseppe Poli[a], Fiorella Biasi[a].

[a]Department of Clinical and Biological Sciences, University of Turin, 10043 Orbassano (Turin), Italy.

Authors' e-mail addresses: Noemi Iaia: noemi.iaia@unito.it; Daniela Rossin: d.rossin@unito.it; Barbara Sottero: barbara.sottero@unito.it; Ivana Venezia: ivana.venezia@edu.unito.it; Giuseppe Poli: giuseppe.poli@unito.it; Fiorella Biasi: fiorella.biasi@unito.it

§Corresponding author:

Prof. Fiorella Biasi

Dept. of Clinical and Biological Sciences, University of Turin

San Luigi Hospital, Regione Gonzole 10,

10043 Orbassano (Turin), Italy

Tel.: 0039-011-6705420

Fax: 0039-011-6705424

Email: fiorella.biasi@unito.it

Abstract

The alteration of the intestinal barrier function is currently believed to be involved in the pathogenesis of gut diseases mainly associated with the activation of inflammation processes. Diet plays an important role in the control of human gut integrity. Theobromine is a natural methylxanthine present in dark chocolate particularly abundant in cocoa bean shell. This is a polyphenol rich by-product generated in cocoa industrial processing, which is gaining value as a functional ingredient. This study aims to highlight for the first time the capability of theobromine in protecting the intestinal cell monolayer from a mixture of dietary oxysterols showing an inflammatory action in terms of IL-8 and MCP-1 overproduction. Differentiated CaCo-2 cells were treated with 60 µM oxysterol mixture and pre-incubated with 10 µM theobromine. Intestinal barrier damage was investigated in terms of tight junction claudin 1, occludin and JAM-A protein levels, matrix metalloproteinase (MMP-2 and MMP-9) activation and anti/pro-apoptotic protein changes. The observed cell monolayer permeability protection by theobromine may be due to its ability to inhibit the production of cytokines and matrix metalloproteinase that can be responsible for tight junction loss and apoptosis in intestinal cells. Our findings provide additional mechanistic hints on the healthy effect of theobromine cocoa component as an attractive natural molecule in the prevention of inflammatory gut diseases.

Key Words: apoptosis; dietary oxysterols; intestinal inflammation; tight junctions; matrix metalloproteinases; theobromine

List of abbreviations:

α-epox 5α,6α-epoxycholesterol; **β-epox** 5β,6β-epoxycholesterol; **7α-HC** 7α-hydroxycholesterol; **7β-HC** 7β-hydroxycholesterol; **7K** 7-ketocholesterol; **Bax** Bcl-2-associated x; **Bcl-xL** B-cell lymphoma-extra-large; **cAMP** cyclic adenosine monophosphate; **CBS** cocoa bean shell; **DMEM** dulbecco's modified eagle's medium; **DTT** dithiothreitol; **ECL** enhanced chemiluminescence; **ELISA** enzyme-linked immunosorbent assay; **ERK** extracellular signal-regulated kinase ; **FBS** fetal bovine serum; **HRP** horseradish peroxidase; **IBD** inflammatory bowel diseases; **IL** interleukin; **IFN-β** interferon β; **LDH** lactate dehydrogenase; **LDS** lithium dodecyl sulfate; **mTOR** mammalian target of rapamycin; **MMP** metalloproteinase; **MCP-1** monocyte chemoattractant protein; **NADH** nicotinamide adenine dinucleotide; **NF-kB** nuclear factor-kappa B; **Oxy-mix** oxysterol mixture; **PBS** phosphate buffer saline; **PDE** phosphodiesterase**; PI3K** phosphatidylinositol 3-kinase; **RT** room temperature; **SDS** sodium dodecyl sulphate; **ST** standard deviation; **TBS** tris-buffered saline; **TJ** tight junction; **TLR** toll-like receptor; **TNF-α** tumor necrosis factor-α; **TTBS** TBS-Tween 20.

1. INTRODUCTION

The intestinal mucosa is a key element in the maintenance of the human health thanks to its double physical and functional activities. It acts as a semi-permeable barrier allowing the absorption of different molecules, such as water or nutrients [1], and at the same time it plays a leading role in regulating the immune system through the recognition of potentially dangerous dietary antigens and microorganisms [2].

Diet plays an important role in the regulation of the human gut integrity. The dietary changes observed in those countries where Western nutritional lifestyle is progressively being adopted, the introduction of lower fiber intake, refined carbohydrates and higher amounts of animal fats have caused a rise in inflammatory intestinal diseases [3].

Animal fat rich foods make cholesterol and its oxidized compounds (i.e. oxysterols) available to intestinal mucosa, where they can exert a strong inflammatory action [4]. A mixture of dietary oxysterols corresponding to high cholesterol consumption was able to activate matrix metalloproteinases (MMPs) 2 and 9, as well as alter tight junction (TJ) cell distribution and production in the human enterocyte-like CaCo-2 cell monolayer [5]. Dietary oxysterols were found to induce inflammation in the same cell model by activating the immune system-related pattern recognition receptors Toll-like Receptor (TLR) 2 and 4 [6].

Notably, dietary guidance from International Organization for the Study of Inflammatory Bowel Diseases (IBD) recommended the importance of a balanced nutritional support aimed at maintaining energy requirement and reducing pro-inflammatory foods [7]. The Mediterranean diet is gaining new consideration because of its high consumption of vegetables and fruits, and low consumption of saturated and trans-fats. It has been associated with a lower risk of IBD onset, disease activity improvement and inflammatory marker decrease [8, 9].

The majority of scientific literature aimed at elucidating the Mediterranean diet health benefits is focused on polyphenol-rich plant foods, which are well-known for their strong antioxidant and anti-inflammatory properties concurring in the prevention of intestinal barrier damage [10-13].

Previous studies reported the ability of phenolic extracts from wine and olive oil in negatively modulating inflammatory-related signaling pathways activated by oxysterols in differentiated enterocyte-like CaCo-2 cells [14-16].

Extracts from cocoa bean shell (CBS) - the main by-product generated in cocoa industrial processing - have recently shown different analytical recovery of phenolic and methylxanthine compounds depending on their fractionation. Their antioxidant and anti-inflammatory properties were tested in this study. CBS fractions rich in (−)-epicatechin and tannins were highlighted for their antioxidant capacity and ability to prevent TLR activation and interleukin (IL)-8 increase upon CaCo-2 cell treatment with a mixture of dietary oxysterols. However, also that CBS fraction containing high amounts of theobromine showed significant anti-inflammatory effects. This finding suggests that theobromine could concur with polyphenol CBS compounds in preserving intestinal damage [6].

Theobromine (3,7-dimethylxanthine) is a natural compound belonging to the family of purine-based natural heterocyclic alkaloids generated from xanthine methylation. Theophylline, caffeine and theobromine are the most known methylxanthines present in plants such as *Camellia sinensis L.*, *Coffea sp*. and *Theobroma cacao L.* used for the production of tea, coffee, and chocolate [17]. They are known for their action as adenosine receptor antagonists and non-selective phosphodiesterases (PDEs) inhibitors on the Central Nervous System. They have been widely used for their bronchodilator - in particular theophylline - neurostimulatory and psychoactive actions (in particular caffeine) [18, 19].

The main source of theobromine is dark chocolate and is also particularly abundant in CBS. Together with caffeine, it accumulates in the cotyledons of cocoa beans and crosses from the seed into the shell during cocoa fermentation. Theobromine is present in this fraction in a concentration 5-7-fold higher than caffeine [20].

Compared with other methylxhantines, theobromine effects have been less studied. Similarly to other xanthine derivatives, theobromine possesses smooth-muscle relaxant, diuretic and coronary

vasodilator properties. Theobromine is used as a natural therapeutic compound in asthma and in other respiratory tract problems [21]. Experiments performed in guinea-pigs showed that the preventing action against persistent cough by theobromine was related to its ability to inhibit improper excitation of the vagus nerve [22].

Noteworthy, theobromine anti-inflammatory potential has also been suggested. As a non-selective PDE inhibitor, it could be responsible for intracellular cyclic adenosine monophosphate (cAMP) level increase, protein kinase A activation and tumor necrosis factor (TNF)-α inhibition [23]. This molecule also displays anti-tumor effect in human glioblastoma cells by attenuating extracellular signal-regulated kinase (ERK) activity, phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) and nuclear factor-kappa B (NF-kB) inflammatory signaling axis [24]. Theobromine antioxidant and anti-inflammatory action independently from its ability to block adenosine receptor pathway has also been suggested [21].

To date, the possible theobromine beneficial effects on the intestine are not yet known.

The aim of our current investigation is to give *in vitro* evidence of theobromine ability in preventing the intestinal mucosal layer derangement induced by a mixture of oxysterols present in a hyper-cholesterol diet. In particular, this cocoa bean compound can decrease inflammatory cytokines, IL-8 and monocyte chemoattractant protein (MCP)-1, and matrix metalloproteinases activated by oxysterols at cellular level. One of the mechanisms by which theobromine maintains epithelial barrier integrity could be associated to its ability to suppress the apoptotic program triggered by oxysterols. Our findings provide additional mechanistic hints on the healthy effect of theobromine as an attractive natural molecule in the prevention of inflammatory gut diseases.

2. MATERIALS AND METHODS

2.1. Materials

Unless otherwise specified, all reagents and chemicals were obtained from Sigma Aldrich Srl (Milan, Italy).

Human IL-8 and MCP-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from PeProtech (DBA Italia Srl, Segrate, Milan, Italy).

Rabbit anti-JAM-A (SC-25629), mouse anti-occludin (SC-133256), mouse anti-claudin 1 (SC-166338), mouse anti- B-cell lymphoma (Bcl)-xL (SC-8392) and mouse anti-Bcl-2-associated x (Bax) (SC-7480) polyclonal primary antibodies were from Santa Cruz Biotechnology (DBA Italia Srl, Segrate, Milan, Italy). Anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody (7074S) and anti-mouse IgG HRP- conjugated secondary antibody (7076S) were from Cell Signaling Technology (Euroclone SpA, Milan, Italy). Lithium dodecyl sulfate (LDS) Sample Buffer 4X and dithiothreitol (DTT) Sample Reducer 10X were purchased from Thermo Fisher Scientific (Life Technologies Italia, Monza, Italy). Proteases inhibitors cocktail "cOmplete ULTRA Tablets Mini EASYpack" and the nicotinamide adenine dinucleotide (NADH) were obtained from Roche SpA (Monza, Italy). Bio-Rad protein assay dye reagent and enhanced chemiluminescence (ECL)® Western Blotting System were from Bio-Rad Srl (SIAL, Rome, Italy). Hybond ECL nitrocellulose membrane was from GE Healthcare Srl (Milan, Italy).

Dulbecco's modified Eagle's medium (DMEM) with high glucose content, fetal bovine serum (FBS) and trypsin solution (trypsin 5g/L) were obtained from Euroclone SpA (Milan, Italy).

Oxysterols [5α,6α-epoxycholesterol (α-epox); 5β,6β-epoxycholesterol (β-epox); 7-ketocholesterol (7K);7α-hydroxycholesterol (7α-HC); 7β-hydroxycholesterol (7β-HC)] were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Theobromine (≥98.0%) was from Fluka (Milan, Italy).

2.2. Cell culture and treatments

Human colorectal adenocarcinoma CaCo-2 cells were provided by the Cell Bank Interlab Cell Line Collection (Genoa, Italy). Cells were plated at 1×10^6 /ml density and were cultured in DMEM supplemented with 10% heat inactivated FBS, 1% antibiotic/antimycotic solution (100 U/ml penicillin, 0.1mg/ml streptomycin, 250 ng/ml amphotericin B and 0.04 mg/ml gentamicin) at 37

°C under 5% CO_2 humidified atmosphere. After reaching 100% confluence, cells were grown for additional 18 days in order to allow their spontaneous differentiation into enterocyte-like phenotype.

Before each treatment, differentiated CaCo-2 cells were maintained in serum-free medium overnight to make them quiescent, then placed in 1% FBS DMEM. Cells were pre-incubated or not with theobromine for 1 h (concentrations are reported below in the analysis sections), and treated with a mixture of dietary oxysterols (Oxy-mix) at 60 µM final concentration at 37 °C for 24 h. The percentage composition of oxysterols used in the Oxy-mix was: 42.96% for 7K, 32.3% for α-epox, 5.76% for β-epox, 4.26% for 7α-HC, and 14.71% for 7β-HC. The molarity of each oxysterol was calculated as 25.8 µM 7K, 19.4 µM α-epox, 3.4 µM β-epox, 2.6 µM 7α-HC, 8.8 µM 7β-HC by considering their average molecular weight of 403 g/mol [14].

2.3. Cell death evaluation

The extracellular release of lactate dehydrogenase (LDH), which was considered as a parameter of cytolysis, was estimated spectrophotometrically at 340 nm wavelength by recording NADH production/min. Cell death was evaluated in cells treated with 60 µM Oxy-mix and pre-treated or not with theobromine increasing concentrations (10 to 30 µM).

LDH release was expressed as enzyme release percentage into cell culture medium (100% cell lysis was obtained by 0.5% Triton X-100 addition to cell plate containing the same cell density as treated cells). Untreated cells were considered as controls.

2.4. Evaluation of cytokines protein levels by ELISA

At the end of each treatment, the evaluation of IL-8 and MCP-1 protein levels was performed in the cell incubation medium by using commercial ELISA kits and following the manufactures instructions. The concentration of the two cytokines was evaluated in a 96-multiwell plate reader (Model 680 Microplate Reader, Bio- Rad laboratories Srl, Milan, Italy) by using a 450 nm

wavelength filter. In each sample the optical density at 655 nm wavelength was recorded as value reference.

The protein levels in the cell medium were estimated by using the Bio-Rad protein assay dye reagent [25]. Data were elaborated with SlideWrite Plus software (Advanced Graphics Software, Rancho Santa Fe, CA, USA), and cytokine concentrations were expressed as pg/mg protein.

2.5. Immunoblotting

At the end of each treatment, cells were scraped and washed with one ml of ice-cold phosphate buffer (PBS). For protein extraction 150 µl of lysis buffer [PBS supplemented with 1% Triton X-100 (v/v), 1% sodium dodecyl sulfate (w/v) (final volume)] were added to each sample. Lysates were incubated for 30 min on ice and centrifuged at 12,000 g for 15 min. Total cell extract protein concentration was evaluated with Bio-Rad protein assay dye reagent.

All samples, containing 50 µg total proteins, were boiled in the Sample Buffer at 100 °C for 5 min (LDS Sample Buffer 4X and DTT Reducer 100X). Boiled samples were subjected to electrophoretic migration on 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel and proteins were transferred to Hybond ECL nitrocellulose membranes.

After protein transfer, the membranes were incubated in TTBS blocking buffer [TTBS: tris-buffered saline (TBS) supplemented with 0.05% (v/v) Tween 20] plus 5% (w/v) skimmed milk powder (final volume) at room temperature (RT) for 1 h.

For tight junction protein detection, membranes were then incubated at 4 °C overnight with either mouse anti-claudin 1 (1:800 dilution), mouse anti-occludin (1:500 dilution), or rabbit anti-JAM-A (1:200 dilution) polyclonal antibodies in TBS containing 0.1% Tween-20 (v/v) and 5% skimmed milk powder (w/v).

Immunoblotting technique was also used to analyze apoptotic protein levels. In this case, the membranes were incubated at 4 °C overnight with mouse anti-Bcl-xL (1:600 dilution) or mouse

anti-Bax (1:200 dilutions) polyclonal antibodies in TBS containing 0.1% Tween-20 (v/v) and 5% skimmed milk powder (w/v).

All the blots were washed twice in TTBS and incubated with anti-rabbit or anti-mouse HRP-conjugated IgG (1:1000 dilutions) in TBS with 0.1% Tween-20 (v/v) and 5% skimmed milk powder (w/v) for 1 h. At the end of this incubation time the membranes were washed as previously described.

The kit Clarity Western ECL and the ChemiDoc™ Touch Imaging System machine (Bio-Rad laboratories Srl, Segrate, Italy) were used to detect chemiluminescence. Densitometric measurements were conducted using software Image J data processing (Bethesda, Maryland, USA). Protein levels were expressed as percentage of the controls (untreated cells). Apoptotic proteins were also shown as Bax/Bcl-xL ratio expressed as fold increase versus control.

2.6. Gelatin Zymography

MMP-2 and MMP-9 activities were evaluated in the cell medium by gel zymography. For this analysis, 8 µl of LDS Sample Buffer 4X were added to each protein sample (20 µg proteins). Samples were used in non-reducing conditions, i.e. in absence of heating and reducing agents, then subjected to electrophoretic migration on 8% SDS-PAGE gel containing gelatin (0.8 mg/ml) with a constant voltage of 110V. Gelatin is one of the most frequently used substrate for MMPs. After electrophoretic migration, the gel was washed with Tris buffer (2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.5, final solution) at RT for 1 h and incubated at 37°C overnight in proteolysis buffer (40 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl2, 0.02% NaN3, pH 7.5, final solution). The gel was then stained for 3 h with Coomassie Blue solution (0.05% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid, final solution) and finally destained with 5% methanol and 7% acetic acid (final solution). The resulting destained bands corresponding to MMP proteolytic activities look clear over a deep blue background. The activity of the two MMPs was estimated by

densitometric analysis using Image J software (Bethesda, Maryland, USA) and expressed as fold increase versus control.

2.7. Statistical analyses

Results were expressed as mean ± Standard Deviation (SD) and data were analysed with GraphPad InStat software (San Diego, CA, USA). Statistical differences among experimental data were evaluated by using the one-way ANOVA test associated with Bonferroni's multiple comparison post-test.

3. RESULTS

3.1. Effects of different theobromine concentrations on cell death

In order to investigate whether theobromine exerts cytotoxic effect on differentiated CaCo-2 cells in the presence or absence of Oxy-mix, the percentage of lactate dehydrogenase cell release was detected. Cells were incubated with increasing concentrations of theobromine (10, 15, 20, 25 or 30 µM) for 24 h. LDH % increase up to 10% was not considered as cytotoxic. All theobromine concentrations did not exert any necrogenic effect whether the compound was added alone or with Oxy-mix. Notably, the enzyme percentage release was found higher in 60 µM Oxy-mix alone than in theobromine + Oxy-mix-treated cells (in particular, both 10 and 15 µM theobromine concentrations) (Table 1). Based on this observation, all subsequent experiments were performed by using 10 µM theobromine.

LDH (% cell relase)		
	-	**Oxy-mix**
Control	2.0 ± 0.05 ##	8.3 ± 0.5 **
10 µM theobromine	2.4 ± 0.05 ##	5.2 ± 0.1 *,#
15 µM theobromine	2.3 ± 0.2 ##	5.1 ± 0.1 *,#
20 µM theobromine	2.8 ± 0.1 ##	5.7 ± 0.2 *,#
25 µM theobromine	2.9 ± 0.03 ##	5.8 ± 0.02 *,#
30 µM theobromine	3.5 ± 0.1 ##	6.3 ± 0.1 *,#

Table 1. Cell viability evaluation of different concentrations of theobromine

LDH release was evaluated in the culture media of differentiated CaCo-2 cells pre-treated or not with different concentration of Theobromine and incubated with 60 µM Oxy-mix for 24h.

LDH was calculated as percentage referred to 100% cell enzyme released into the medium following the addition of 0.5% Triton X-100 to cultured cells grown at the same density of other samples.

Data are reported as means ± SD of three independent experiments. Significantly different vs. controls: *p<0.05; **p<0.01; significantly different vs. Oxy-mix: # p<0.05, ## p<0.01.

3.2. Oxy-mix-dependent IL-8 and MCP-1 increased levels observed in differentiated CaCo-2 cell medium were reduced by theobromine pre-treatment

Theobromine anti-inflammatory property was evaluated in terms of IL-8 and MCP-1 release in the incubation medium of differentiated CaCo-2 cells (Figure 1).

The oxysterol mixture induced a strong increase in the production of both pro-inflammatory cytokines. Cell pre-treatment with 10 µM theobromine was able to decrease the cytokine overproduction induced by the Oxy-mix treatment. IL-8 and MCP-1 medium protein levels in the presence of theobromine alone were similar to those evaluated in untreated cells (control).

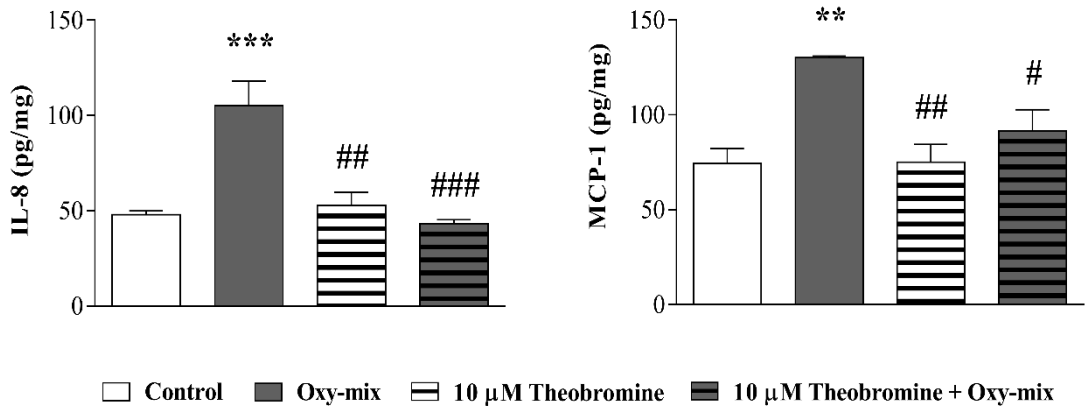


Figure 1. Effect of theobromine on inflammatory marker level increase by Oxy-mix cell incubation.

Differentiated CaCo-2 cells were pre-treated or not with theobromine (10 μM) for 1 h and incubated with 60 μM Oxy-mix for 24 h. IL-8 and MCP-1 protein levels were quantified by ELISA in the cell culture medium. Theobromine was able to preserve the Oxy-mix-dependent cytokine protein level increase in the cell medium. Values are shown as pg chemokine release / cell incubation medium mg protein and are referred as means ± SD of three independent experiments. Significantly different vs. controls: **p<0.01, ***p<0.001; significantly different vs. Oxy-mix: # p<0.05, ## p<0.01, ### p<0.001.

3.3. Theobromine counteracts tight junction impairment dependent from Oxy-mix cell treatment

Claudin 1, JAM-A and occludin were considered as tight junction proteins involved in the regulation of the intestinal layer permeability.

Cell protein levels of these three TJs were evaluated in order to clarify the potential beneficial effect of theobromine in preserving the integrity of the intestinal epithelial barrier.

Western blotting analyses performed in differentiated CaCo-2 cell monolayers treated with Oxy-mix for 24 h showed a significant decrease in all TJ protein levels. On the contrary, cell pre-incubation with 10 μM theobromine for 1 h attenuated the Oxy-mix-dependent decrease in TJ protein levels. In particular, Claudin 1 and JAM-A amounts similar to controls were observed (Fig.2).

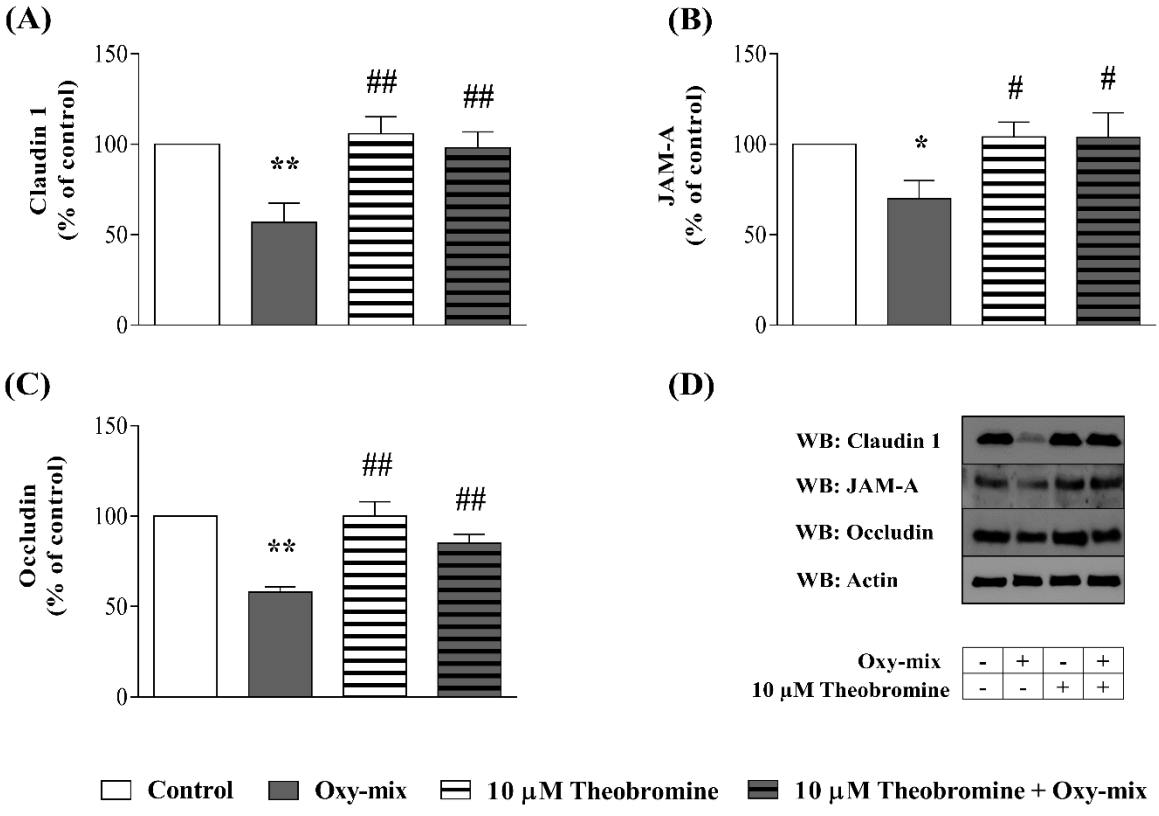


Figure 2. Tight Junction protein levels in differentiated CaCo-2 cells treated with Oxy-mix, in the presence or absence of theobromine.

Decreased protein levels of claudin 1, JAM-A and occludin were evaluated in lysates from CaCo-2 cells incubated with 60 µM Oxy-mix for 24 h; 10-µM theobromine-pre-treated cells for 1 h protected oxysterol-mediated TJ loss. Data are expressed as percentage of control (considered as 100%). Values are means ± SD of three independent experiments. Significantly different vs. controls: *p<0.05, **p<0.01; significantly different vs. Oxy-mix: # p<0.05, ## p<0.01.

3.4. Cell release of active MMP-2 and MMP-9 is prevented by theobromine.

MMPs are zinc-dependent enzymes, which play a key role in modifying cellular adhesion and motility thus concurring to epithelial barrier destabilization. MMP-2 and MMP-9 were evaluated by direct gelatin zymography in the cell medium, where their activated form is present.

The treatment of differentiated CaCo-2 cells with 60 µM Oxy-mix allowed to observe the induction of both MMP-2 and -9 activities compared to controls (Fig.3).

Cell pre-incubation with theobromine brought back MMPs activities to control values, thus strengthening its protective role against Oxy-mix-dependent damage on cell monolayer.

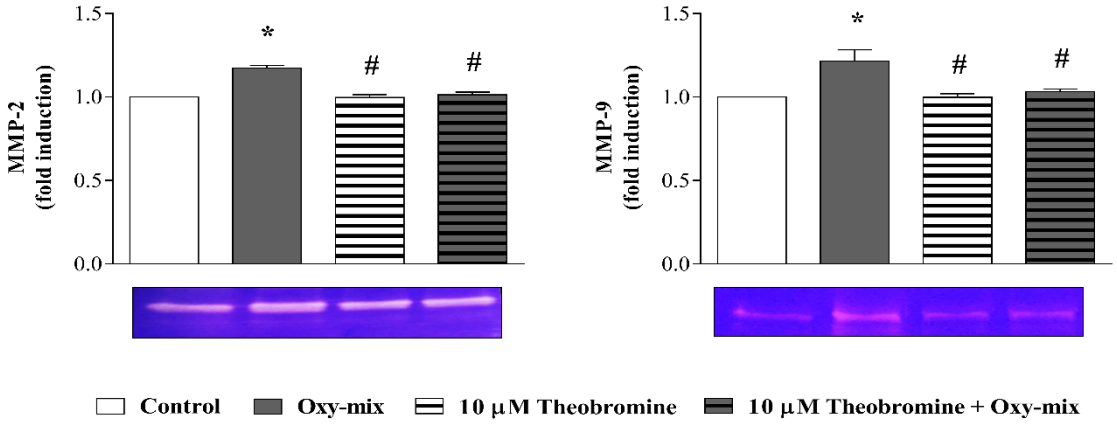


Figure 3. MMP-2 and MMP-9 induction in differentiated CaCo-2 cells treated with Oxy-mix. Effect of theobromine.

The induction of MMP-2 and MMP-9 was evaluated by gel-zymography in differentiated CaCo-2 cells after 60 µM Oxy-mix-treatment for 24 h. Theobromine pre-treatment reduces MMPs' activation to normal values. All data represent means ± SD of three independent experiments. Significantly different vs. controls: *$p<0.05$; significantly different vs. Oxy-mix: #$p<0.05$.

3.5. Theobromine may play a role in the maintenance of the epithelial barrier integrity by regulating apoptosis.

Excessive proteolysis together with TJ protein damage, as well as the activation of intestinal epithelial cell apoptosis can concur in affecting barrier function. We focused our attention on the apoptosis induced by the Oxy-mix and the possible protective effect of theobromine by evaluating cell protein level changes in pro-apoptotic Bcl-2 associated X (Bax) and anti-apoptotic B-cell lymphoma-extra-large (Bcl-xL).

Differentiated CaCo-2 cells were pre-treated or not with theobromine for 1h and treated with Oxy-mix for 24h. Cell incubation with 60 µM Oxy-mix significantly increased pro-apoptotic Bax protein levels (Fig. 4A), while markedly reduced anti-apoptotic Bcl-xL protein levels (Fig. 4B). The

significant increased Bax/Bcl-xL protein level ratio versus control (considered as 1) confirmed the activation of the apoptotic pathway by the Oxy-mix (Fig. 4C).

On the other hand, oxysterol-dependent apoptotic protein changes were reversed when cells were pre-incubated with 10 µM theobromine, thus supporting a further beneficial role of this molecule in exerting anti-apoptotic activity in intestinal cell monolayer.

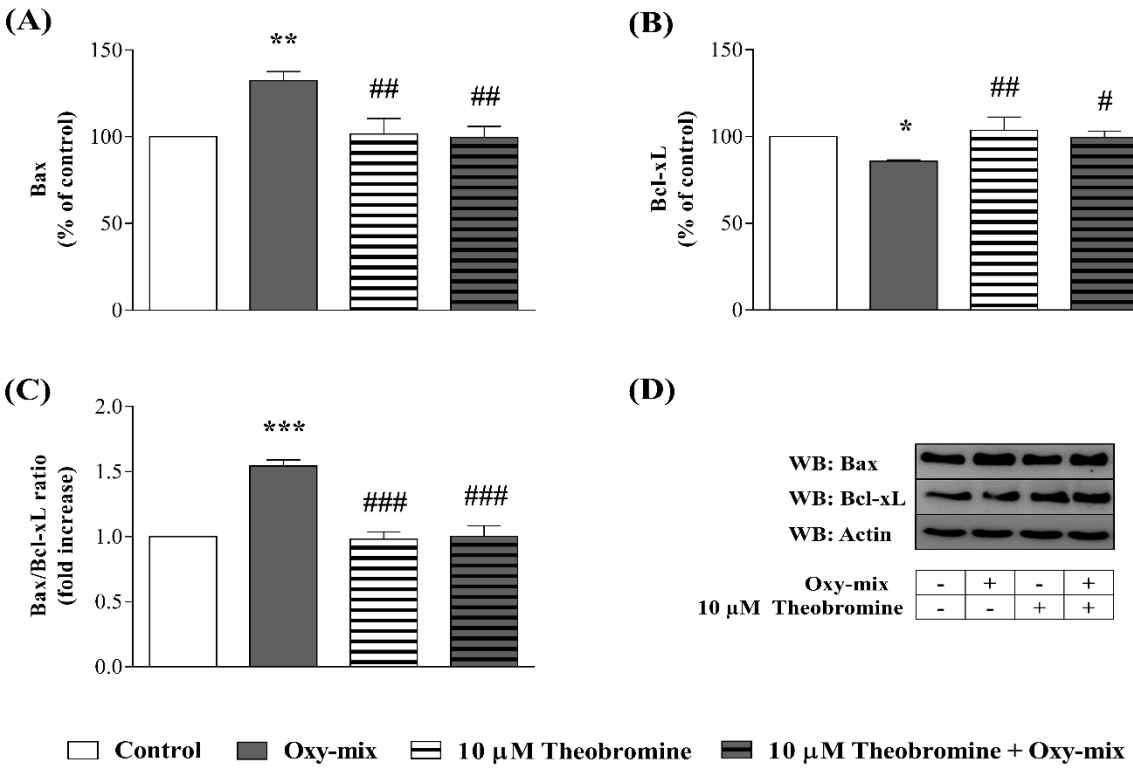


Figure 4. Theobromine restores apoptotic protein levels impaired by Oxy-mix cell treatment.

Oxy-mix cell incubation reduced anti-apoptotic Bcl-xL, while markedly increased pro-apoptotic Bax proteins. One-hour cell pre-treatment with theobromine (10 µM) prevented changes of the two above-mentioned proteins involved in the apoptosis modulation. Bax (A) and Bcl-xL (B) cell protein levels were determined by Western Blotting in differentiated CaCo-2 cells treated with Oxy-mix for 24 h pre-treated or not with theobromine. Bax/Bcl-xL ratio is expressed as fold increases versus control (C). Representative immunoblots of Bax, Bcl-xL, and actin as reference protein are shown (D). All values represent means ± SD of three independent experiments. Significantly different vs. controls: *p<0.05, **p<0.01, ***p<0.001; significantly different vs. Oxy-mix: #p<0.05, ## p<0.01, ### p<0.001.

4. DISCUSSION

The alteration of the intestinal barrier function is currently believed to play an important role in the pathogenesis of gut diseases mainly associated with the activation of inflammation processes. The mucosa layer integrity loss could allow dietary/bacteria-derived molecules triggering uncontrollable inflammatory signalling cascade. The maintenance of barrier integrity is both negatively and positively influenced by eating habits. Nowadays, a rise in Western diet-associated diseases represents a public health problem. Over the last decades, worldwide health organizations adopted many dietary strategies to reduce disease risk or outcomes. In this context, many studies aim to explore and utilize natural compounds providing antioxidant and anti-inflammatory benefits. They represent a possible strategy in attenuating and/or preventing inflammatory diseases, including the intestinal ones.

Cocoa and its related products like chocolate are widely consumed. Several studies demonstrated its beneficial effects as functional food, especially related to the high content in antioxidant flavanols - particularly epicatechin and procyanidins - and methylxanthines.

Theobromine is the main methylxanthine found in cocoa and cocoa-containing foods. It is present in high amounts in the external tegument covering cocoa beans known as cocoa bean shell, a by-product with great bio-functional potential that has been raising attention in industrial policies to develop circular economy strategies [20].

Theobromine is often associated with chocolate positive effect on cognitive performance and mood. In addition to that, its anti-inflammatory and anti-tumoral properties, as well as cardiovascular disease risk protection have been observed [21,26].

In the present study we demonstrated theobromine ability to prevent intestinal damage induced by dietary oxysterols in differentiated CaCo-2 cell monolayer, which represents an established model of intestinal barrier. The oxysterols used in our experiments were added to the cells in mixture and concentrations as they can be detectable in cholesterol-rich foods. In agreement with other studies on differentiated CaCo-2 cells, they showed remarkable pro-inflammatory effects in terms of IL-8

and MCP-1 production, and MMP induction. Deiana and colleagues demonstrated that MMP activation by the Oxy-mix was closely associated with decreased levels of TJs and increased cell monolayer permeability in differentiated CaCo-2 cells [5]. Notably, a marked increase of MMP-9 was observed in the intestinal tissue of patients with IBD closely associated with increasing intestinal TJ permeability, as also proved in CaCo-2 cells [27].

The anti-inflammatory property of theobromine against Oxy-mix action was demonstrated in our experimental model in terms of IL-8 and MCP-1 decrease in cell culture medium. These two cytokines have been shown to be involved in the induction of inflammatory and immune response when intestinal mucosa is damaged by bacteria toxin exposure [28].

Consistently, theobromine was also able to avoid TJ protein level loss induced by the Oxy-mix. Occludin, claudins and JAM-A are transmembrane proteins, which cooperate in the regulation of paracellular permeability barrier [29]. Proteinase deregulation was suggested to cause increased intestinal barrier damage, which on the other hand, contributes to fueling inflammatory and immune response, a typical feature of chronic diseases such as IBD [30]. Therefore, our observed cell monolayer permeability protection by theobromine could be due to its ability to inhibit the production of cytokines and matrix metalloproteinases in CaCo-2 cells. Theobromine was found to posses anti-inflammatory and antioxidant effects in chondrocytes treated with IL-1β, by suppressing cyclooxygenase-2 , prostaglandin E2, TNF-α , MCP-1, MMP-3 and MMP-13, as well as reactive oxygen species, nitric oxide production and inducible nitric oxide synthase expression.

Theobromine anti-inflammatory action was proposed to be associated with its ability to down-regulate NF-κB activity [31]. Similarly, theobromine was suggested as a possible therapeutic approach in treating obesity for its capacity to reduce MCP-1 and IL-1β associated with the inhibition of pre-adypocyte differentiation into mature adipocytes in an *in vitro* model of fat tissue [26]. Theobromine inhibitory effect on phosphodiesterase-4 dependent-regulatory pathway was suggested as a mechanism of action to reduce obesity in mice [32]. The switch off of inflammation through the inhibition of PDE4 is actually considered as a promising therapeutic target for many

inflammatory diseases [33, 34]. Pentoxifylline, a synthetic methylxanthine widely used as a drug, was observed to decrease MMP-2 activity via TNF-α inhibition in hypertensive rats' aorta [35]. Pentoxifylline action was also attributed to its ability to inhibit Toll-like receptor pro-inflammatory pathway [36]. In our recent study using CaCo-2 cells treated with Oxy-mix, we observed that not only CBS fractions with high quantities of (−)-epicatechin and tannins, but also the CBS fraction mainly containing theobromine were able to prevent TLR2 and 4 activation and TLR downstream effectors' production, i.e. IL-8, interferon (IFN)-β and TNF-α [6].

Increased apoptosis can also play a role in barrier derangement; the excessive lumen cell shedding is associated to inflammatory intestinal disease activity [37, 38]. We demonstrated that Oxy-mix induced apoptosis by enhancing pro-apoptotic Bax and decreasing anti-apoptotic Bcl-xL protein levels in differentiated CaCo-2 cells. In addition to that, theobromine pre-treatment restored apoptotis in the intestinal monolayer by reversing cell protein levels, thus decreasing Bax/Bcl-xL ratio. Pro-inflammatory TNF-α overproduction is considered as one of the factors responsible for excessive epithelial death in IBD patient ileum and colon [39]. We would like to highlight the hypothesis that matrix metalloproteinases, which are responsible for increased intestinal permeability in IBD and experimental colitis [40, 27], may also participate in apoptosis induction as shown in hepatic stellate cells and human cardiac stem cells [41, 42]. On the other hand, cell-cell contacts in primary colon cells prevent apoptosis [43].

Therefore, theobromine anti-apoptotic effect could be due to its ability to decrease the production of molecules directly involved in the apoptotic fate such as TNF-α, but also to avoid cell barrier derangement due to matrix metalloproteinase activation and TJ loss in an inflammatory environment.

5. CONCLUSIONS

The here reported data underline theobromine ability in preserving intestinal cell monolayer from the damaging action of a mixture of oxysterols detectable in cholesterol rich foods. The main

theobromine action mechanism consists in decreasing specific inflammatory mediators, which destabilize membrane structure integrity. CBS is gaining value as a polyphenol-rich cocoa by-product to be reused as a functional ingredient or cocoa substitute. Theobromine concentration levels are very high in CBS. Therefore, it is reasonable to hypothesize that this molecule can be partly responsible for the benefits associated with cocoa consumption.

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Declarations of interest: none

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Figure1

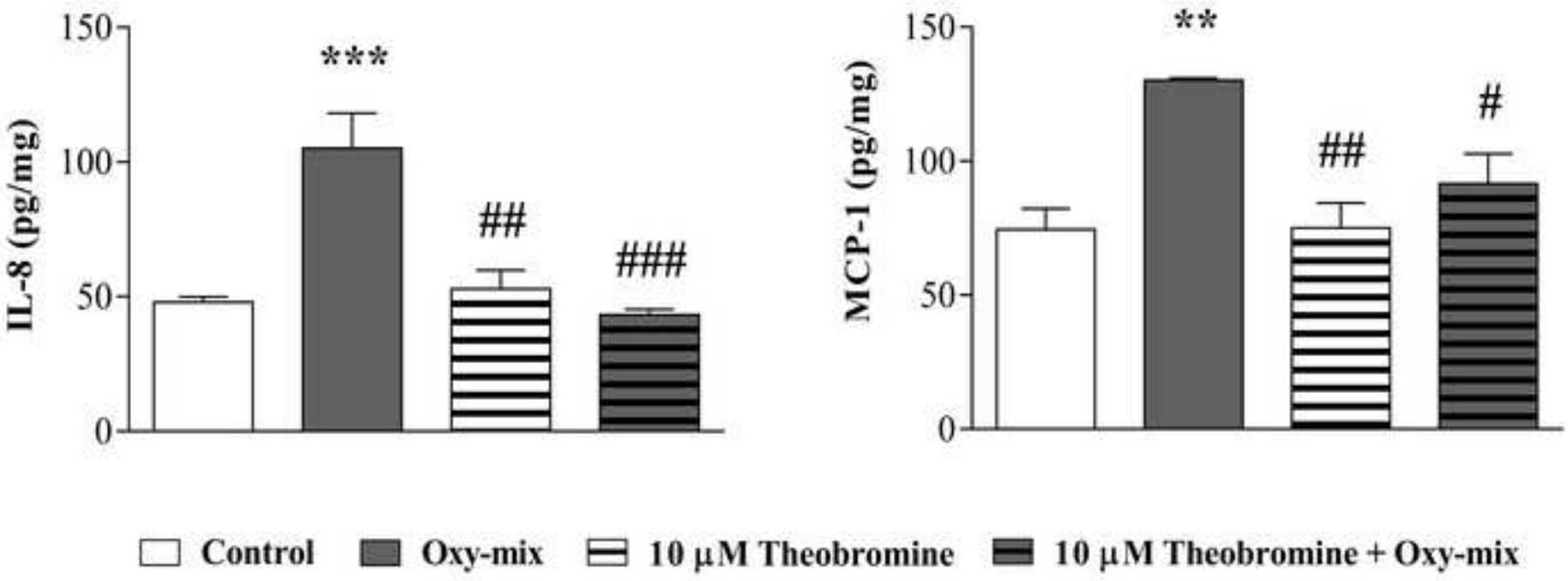


Figure2

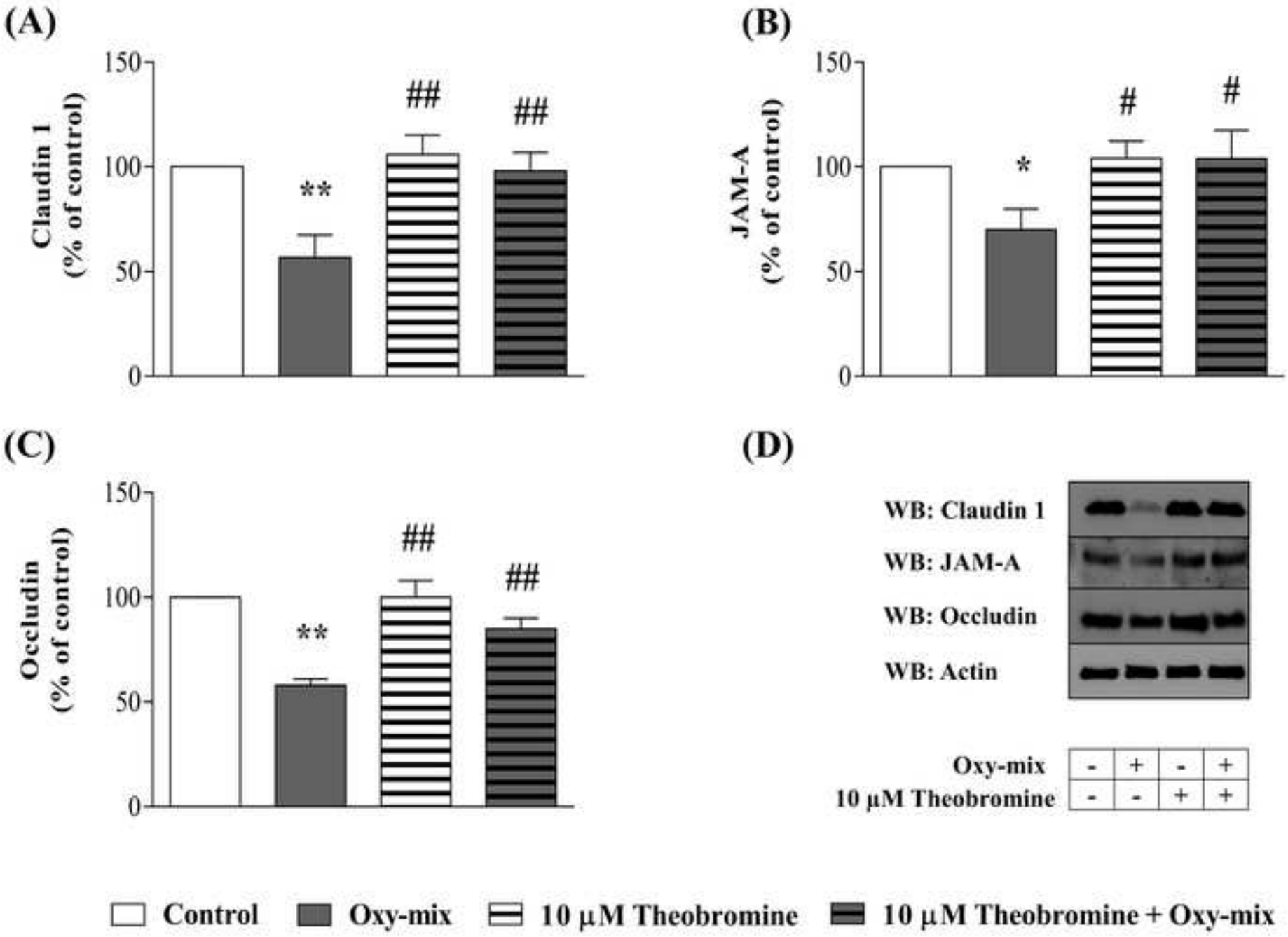


Figure3

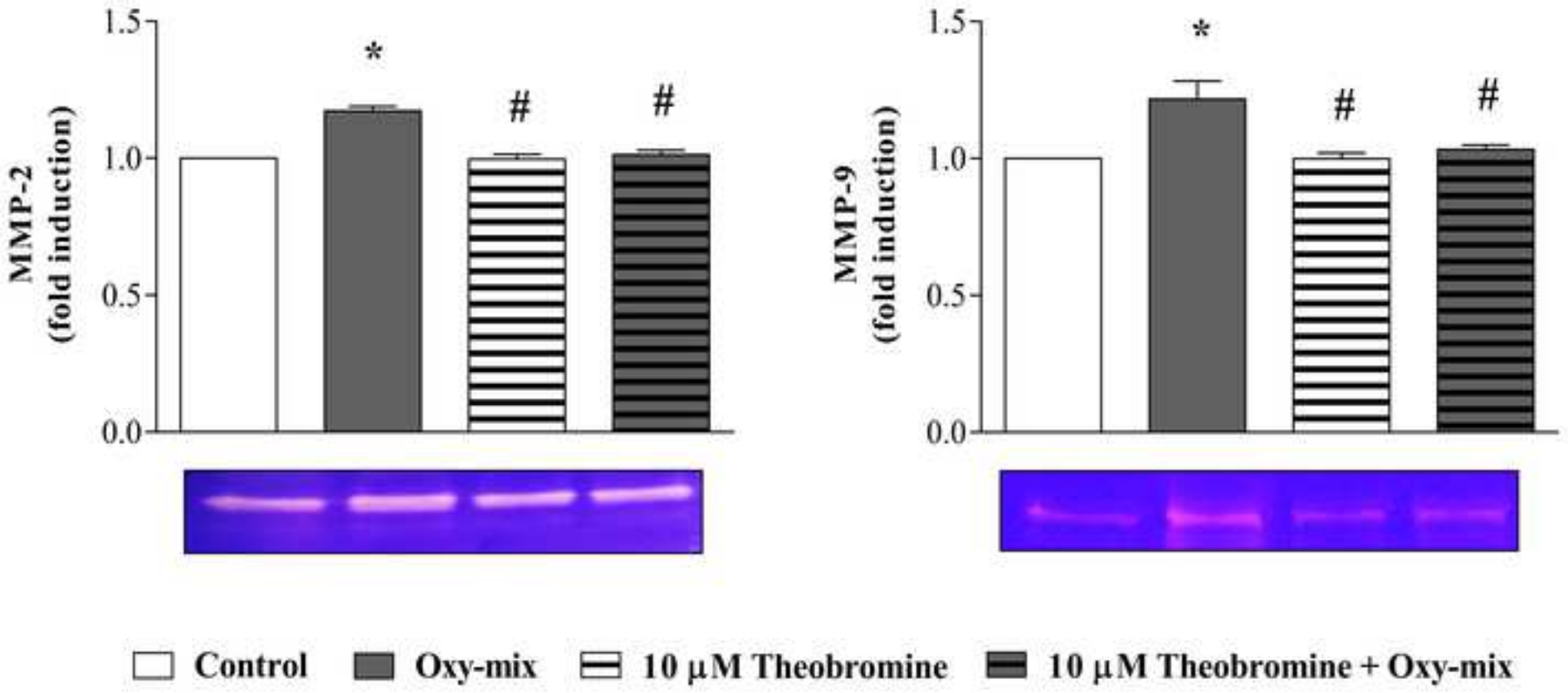
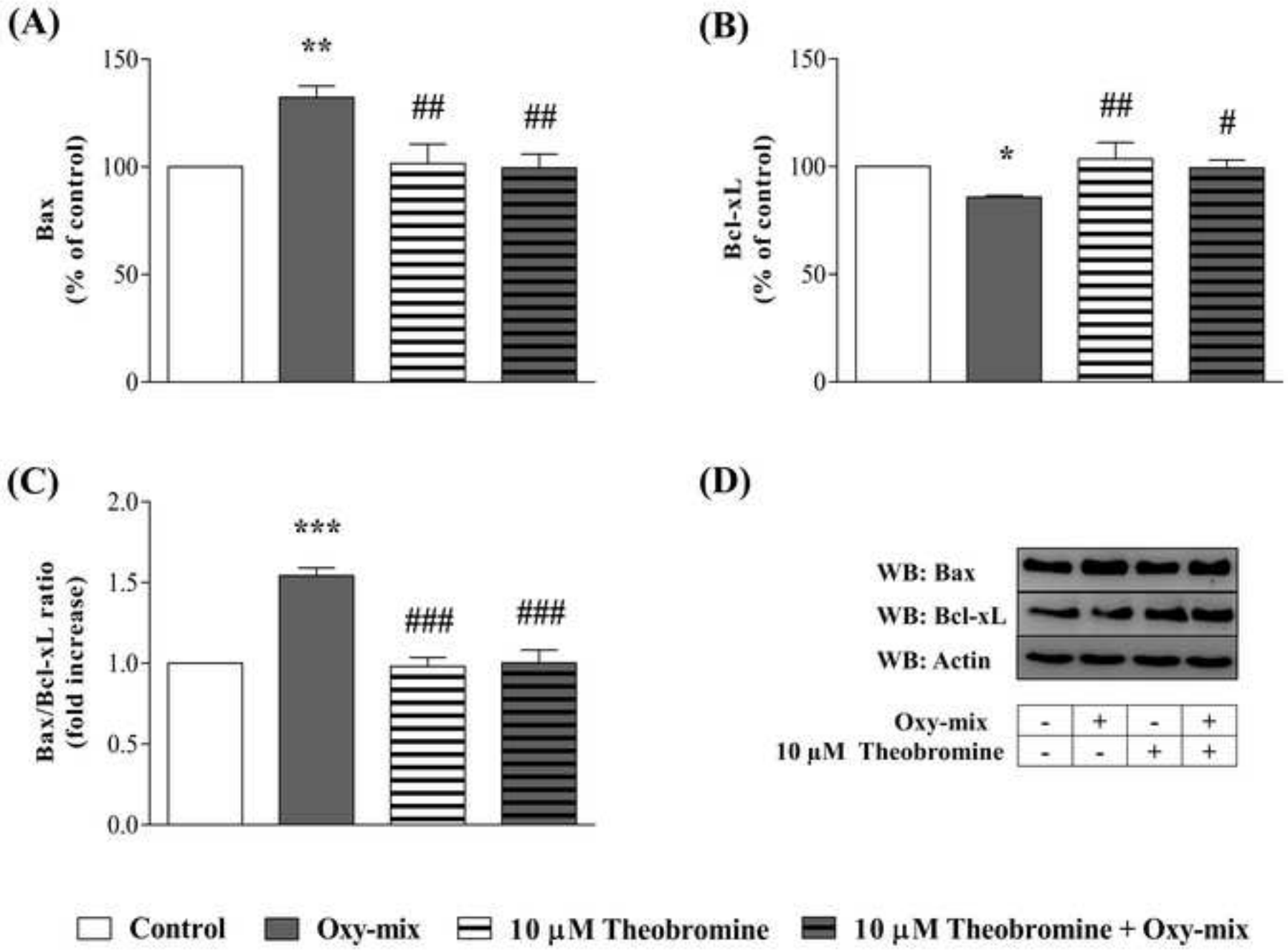


Figure4



Table

Author Contributions

Conceptualization, F.B., N.I.; Funding acquisition, F.B.; Investigation, D.R., N.I., I.V.; Supervision, F.B., G.P.; Validation, B.S.; Writing –original draft, F.B., N.I., I.V.; Writing - review & editing, F.B., N.I.