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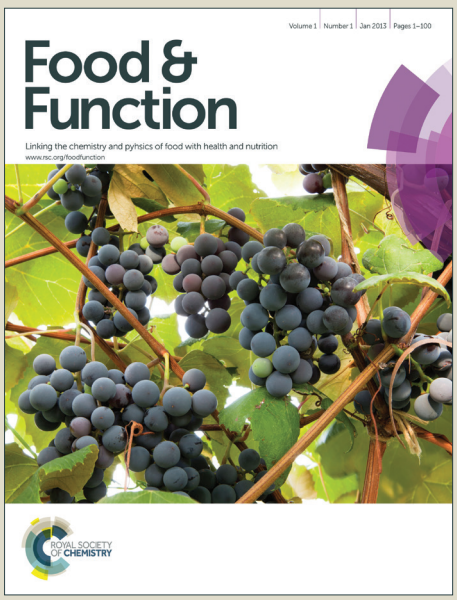

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EFFECT OF *IN VITRO* GASTRO-INTESTINAL DIGESTION ON THE ANTI-INFLAMMATORY ACTIVITY OF VITIS VINIFERA L. LEAVES

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ABSTRACT

Botanicals are widely consumed all over the world for health purposes, with increased usage in the general population, in many different types of products, including foods, and plant food supplements. Several reports support for the beneficial effects of botanicals against gastro-intestinal inflammation. However, no studies regarding the anti-inflammatory activity in the gastrointestinal tract of red vine leaves have been reported so far. The present work investigates the biological activity of *Vitis vinifera* L. water extract (VVWE) from dried leaves in two *in vitro* models of gastric and intestinal inflammation, respectively. The extract was characterized by a validated HPLC-DAD method, and tested on human epithelial gastric (AGS) and intestinal (Caco-2) cells with the aim to investigate the inhibitory effect on IL-8 secretion and promoter activity, before and after *in vitro* gastric or gastro-intestinal digestion. Our results show that the water extract from red vine leaves inhibits TNFα-induced IL-8 secretion and expression in human gastric epithelial cells; the effect should be maintained, although to a lesser extent, after gastric digestion. On the contrary, the effect after intestinal digestion is dramatically decreased since degradation of the active components in the gut does not allow the extract to efficiently counteract TNFα or IL-1β induced IL-8 expression and NF-κB pathway. The main molecular target of VVWE at the gastric level includes TNFα-induced activation of NF-κB and occurs at concentrations easily reachable after PFS consumption based on red vine leaves water extract as ingredient. Our findings suggest that PFS containing water extracts from *Vitis vinifera* L. leaves could be useful to inhibit/attenuate gastric inflammation inhibiting IL-8 secretion and expression through impairment of the NF-κB pathway.

KEYWORDS

Vitis vinifera L. leaves, gastric inflammation, *in vitro* digestion, NF-κB,

Introduction

Gastrointestinal inflammatory diseases are emerging pathological conditions whose prevalence has increased in the last few years; these pathologies, including inflammatory bowel diseases (IBD) in the gut and gastritis and ulcer in the stomach, are strictly connected to the westernization of lifestyle and industrialization [1].

Gastritis is an inflammation of gastric mucosa frequently caused by the presence of the bacterium *Helicobacter pylori* (*H. pylori*). During *H. pylori* infection, gastric epithelial cells show higher levels of cytokines including IL-1β, TNF-α, and IL-8, a potent chemokine playing a key role in gastric diseases. This response is highly dependent by the NF-κB activation, a transcription factor crucial in gastro-intestinal inflammatory diseases [2]. NF-κB is deeply involved in the control of the transcription of several pro-inflammatory mediators, thus leading to the worsening of inflammatory conditions [3]. Moreover, activation of the NF-κB pathway in gastric epithelial cells has been suggested to play a critical role in *H. pylori*-induced chronic inflammation and gastric carcinogenesis [4].

IBD represents a heterogeneous group of gastrointestinal inflammatory disorders, where commensal gut flora provokes an either (a) insufficient or (b) uncontrolled immune response. This results either in a lack of or in excessive inflammation mainly manifesting as Crohn's disease or ulcerative colitis [5]. The intestinal epithelium is crucial for the defence against invading pathogens, and also plays a critical role in the development of the gastrointestinal immune response. It represents a physical barrier to limit pathogen entry into the circulation by forming intercellular tight junctions between epithelial cells [6] [7]. Alterations of the immune response in these cells mediate IBD [8]; as a consequence, these cells can release chemokines and cytokines in response to enteric microbial pathogens and amplify the inflammatory response triggered by immune cells associated with the underlying mucosa [8]. Among the mediators released by epithelial cells, IL-8 holds a pivotal position by promoting the recruitment of immune cells at the site of inflammation [9]. Excessive and prolonged expression of pro-inflammatory mediators could be harmful to the host leading to the

progression of the disease. The search for new compounds able to interfere with these mechanisms by preventing a prolonged inflammation could be useful for human health. Among them, botanicals are widely consumed all over the world for health purposes, with increased usage in the general population, in many different types of products, including foods (teas and juices), plant food supplements (PFS), herbal medicinal products, etc [10] [11]. Several reports, including some from our group, support for the beneficial effects of botanicals against gastro-intestinal inflammation, including olive oil phenols [12] [13], pomegranate (*Punica granatum* L.) [14], green tea (*Thea sinensis* L.) [15] [16] [17], coffee polyphenols [18], and turmeric (*Curcuma longa* L.) [19].

Recently, a consumer survey evaluating the usage of PFS across six European countries, reported *Vitis vinifera* L. in the top list among PFS ingredients and at fifth position in Germany [11]. A recent systematic review aimed to summarize and critically evaluate the clinical evidences for or against the efficacy of PFS or their ingredients for coping inflammatory conditions revealed that only thirteen publications were related to *Vitis vinifera* L.: among them, four studies described the effect obtained with the use of grapes whereas only one dealt with grape leaves [20]. Red vine leaf consists of the dried leaves from cultivars of *Vitis vinifera* L. (Vitaceae) known as teinturiers, and characterized by black grapes and red pulp. According to ESCOP Monography, leaves should contain not less than 4.0 per cent of anthocyanins, expressed as cyanidin-3-O-glucoside [21]. Red vine leaves are ingredients largely diffused in PFS or herbal drugs, mainly in association with other botanicals, for treatment of symptoms of chronic venous insufficiency or problems related to varicosis such as painful and heavy legs. However, no studies regarding the anti-inflammatory activity in the gastrointestinal tract of red vine leaves have been reported so far.

The present work investigates the biological activity of an aqueous extract from *Vitis vinifera* L. dried leaves (VVWE) in two *in vitro* models of gastric and intestinal inflammation, respectively. To achieve this goal, an HPLC-DAD method was validated in order to quantify flavonoids and phenolic acid occurring in the extract. VVWE phytochemically characterized was tested on human epithelial gastric (AGS) and intestinal (Caco-2) cells to investigate the inhibitory effect on IL-8

secretion and promoter activity. To study the molecular mechanisms by which red vine extract may modulate the inflammatory response by gastro-intestinal epithelial cells we considered the NF-κB-signaling pathway and the expression/secretion of IL-8 as a typical NF-κB regulated gene involved in gastro-intestinal inflammation.

To gain new insights on the role of gastro-intestinal digestion against the anti-inflammatory activity of the extract, VVWE was subjected to *in vitro* gastric or gastrointestinal digestion, then the main phenols were characterized and the digested extract was tested on the inflammatory targets as described above.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium/F12 (DMEM)/F12 (1:1), penicillin, streptomycin, L-glutamine, Sodium Pyruvate and trypsin-EDTA were from Gibco (Life Technologies Italia, Monza, Italy). DMEM, MEM non-essential amino acid solution and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Aldrich (Milan, Italy). All reagents used for analytical determinations and for the biological assays were HPLC grade. Human TNFα and IL-1β and Human IL-8 Elisa Development Kit were from Peprotech Inc. (London, UK). Foetal bovine serum (FBS), and disposable material for cell culture were purchased by Euroclone (Euroclone S.p.A., Pero-Milan, Italy). Human adenocarcinoma cells (AGS, CRL-1739) were purchased from LGC Standard S.r.l., Milano, Italy; Caco-2 cells were kindly donated by Dr. Y. Sambuy (INRAN - National Research Institute on Food and Nutrition, Italy). Delfinidin 3-O-glucoside chloride (purity >95%), cyanidin 3-O-glucoside chloride (purity >96%), (Extrasynthese, Genay, France), petunidin 3-O-glucoside chloride (purity >98%), peonidin 3-O-glucoside chloride, (purity >95%), malvidin 3-O-glucoside chloride, (purity >95%), hyperoside (purity >98%), and kaempferol-3-O-glucoside (purity >99%) were purchased from Extrasynthese, (Genay, France); caftaric acid (purity >97%), ferulic acid (purity 99.6 %), chlorogenic acid (purity 99.6 %), caffeic acid (purity 97%), rutin

(purity >97%), quercetin-3-O-glucoside (purity ≥ 98%), quercetin-3-O-glucuronide (purity >98%), and epigallocatechin-3-gallate (purity >99%, EGCG) were from Sigma-Aldrich (St Louis, USA). The plasmid NF-κB-LUC containing the luciferase gene under the control of three κB sites was a gift of Dr. N. Marx (Department of Internal Medicine- Cardiology, University of Ulm, Germany). Native IL-8-LUC promoter was kindly provided by Dr. T. Shimohata (Department of Preventive Environment and Nutrition, University of Tokushima Graduate School, Japan). Britelite™ plus was from Perkin Elmer (Monza, Italy).

Plant material and preparation of the water extract

Dried and powdered leaves from *Vitis vinifera* L. cv. teinturiers, kindly supplied by Phytolab company (Vestenbergsgreuth, Germany), were maintained at 4°C until extraction. VVWE was prepared according to ESCOP monography. Briefly 10 grams of dried leaves of *Vitis vinifera* L. were extracted twice with 100 mL of distilled water for 4 and 16 hours, respectively, at room temperature in dark conditions. The mixture was filtered through a supervelox filter paper in order to remove plant debris, and the aqueous extract obtained was transferred into a flask, frozen in a cold room with dry ice and alcohol and placed at -20°C overnight. VVWE was lyophilized and maintained at -20°C. Recovery (w/w) was 26 %, calculated on the dried drug weight. Before proceeding with the biological evaluation, the extract was dissolved in sterilized distilled water at the concentration of 50 mg/mL, and immediately stored in aliquots at -20°C.

***In vitro* gastro-intestinal digestion**

Gastro-intestinal digestion was simulated using an *in vitro* approach previously described, with minor modifications [22 23]. Briefly, the aqueous extract (600 mg) was incubated for 5 minutes at 37 °C with 6 mL saliva juice (12 mM KCl, 2 mM KSCN, 7.4mM NaH_2PO_4, 4 mM Na_2SO_4, 5 mM NaCl, 1,8 mM NaOH, 3.3 mM urea, 89 µM uric acid, 145 mg/L amylase and 50 mg/L mucin). 12 mL gastric juice (47 mM NaCl, 2.2 mM NaH_2PO_4, 11 mM KCl, 2.7 mM $CaCl_2$, 5.7 mM NH_4Cl, 8.3 mL/L HCl, 3.6 mM glucose, 0.1 mM glucuronic acid, 1.4 mM urea, 1.5 mM glucosamine hydrochloride, 1g/L BSA, 1g/L pepsin and 3 g/L mucin) was added to the suspension and the

sample was incubated for 2 hours at 37°C. At the end of the incubation, the digested sample was frozen and lyophilized. The remaining suspension was treated with 12 mL of duodenal juice (120 mM NaCl, 40 mM $NaHCO_3$, 0.6 mM KH_2PO_4, 7.6 mM KCl, 0.25 mM $MgCl_2$, 0.18 mL/L HCl, 1.36 mM $CaCl_2$, 100 mg/L urea, 1 g/L BSA, 3 g/L pancreatin and 0.5 g/L lipase) and 6 mL of bile juice (90 mM NaCl, 69 mM $NaHCO_3$, 5 mM KCl, 0.2 mL/L HCl, 1.5 mM $CaCl_2$, 250 mg/L urea, 1.8 g/L BSA, 6 g/L bile). After an incubation of 2 hours at 37°C under constant agitation, the sample was centrifuged for 5 minutes at 3000 g and the supernatant, simulating the intestinal digested extract, was frozen and then lyophilized. All the samples were then stored at -20°C until use.

HPLC-DAD optimization and validation

Two HPLC methods using a Diode Array Detector (DAD) have been set up and validated according to FDA Guidelines on Bioanalytical Method Validation [24]. Calibration curves were prepared plotting the peak areas of each analyte *vs.* the corresponding concentration and fitted by least-squares linear regression. Each standard solution was prepared in 0.1 N HCl and analyzed in three separated chromatographic runs. Linearity of the calibration curves was assessed by the correlation coefficient R^2 (**Table 1).**

For each standard, the limits of detection (LOD) and quantification (LOQ) were established at a signal-to-noise ratio of 3 and 10, respectively, using ChromeQuest software. Intra-day precision was determined by calculating the variation coefficient (RSD%) of the peak areas of five replicates injected in the same day. Inter-day precision was evaluated by repeating the intra-day precision study in five different days.

The methods were applied for the determination of anthocyanins (delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside), flavonols (rutin, hyperoside, quercetin-3-O-glucoside, quercetin-3-O-glucuronide, kaempferol-3-O-glucoside) or caffeic acid derivatives (caftaric, ferulic, chlorogenic and caffeic acids). The chromatographic column used for anthocyanins determination was a Synergi 4u MAX-

RP 80A (250x4.60 mm 4 µm) (Phenomenex, Torrance, CA, USA). The analysis was performed using a gradient elution at a flow rate of 0.8 mL/min, where: A) water:acetonitrile:formic acid 87:3:10 (v/v/v); and B) acetonitrile:water:formic acid 50:40:10 (v/v/v). The gradient was programmed as follows: 0-15 min: 94-70 % A, 15-30 min: 70-50 % A, 30-35 min: 50-10 % A, 35-38 min: 10% A isocratic, 38-48 min: 10-94% A. The detection was set at 520 nm.

The chromatographic column used for for quantifying flavonols and caffeic acid derivatives was a Synergi 4u MAX-RP 80A (250x2 mm 4 µm) (Phenomenex, Torrance, CA, USA). The analysis was performed using a gradient elution at a flow rate of 0.3 mL/min, where: A) water:acetonitrile:formic acid 96.9:3:0.1 (v/v/v); and B) acetonitrile:water:formic acid 50:49.9:0.1 (v/v/v). The gradient was programmed as follows: 0-15 min: 94-70 % A, 15-30 min: 70-50 % A, 30-35 min: 50-10 % A, 35-38 min: 10% A isocratic, 38-48 min: 10-94% A. The detection was set at 360 nm.

Iso-Disc Filters PTFE 0.45 µm were from Supelco Analytical (Bellefonte, PA, USA). The HPLC equipment was from Thermo (San Josè, CA, USA) and consisted of a pump (P2000, Thermo Separation products, San Josè, CA, USA), an interface (SN4000, Thermo Separation products, San Josè, CA, USA) UV detector (975-UV), a Diode Array Detector (6000 LP, Thermo Separation products, San Josè, CA, USA, an injection valve (Rheodyne, Cotati, CA, USA) with a 20 µL loop.

Total phenol content and anthocyanin assays

Total polyphenol content was determined according to the Folin-Ciocalteau's method, as reported by Singleton and Rossi [25]. Total anthocyanin content was evaluated by using the pH differential method, based on the property of monomeric anthocyanins to reversibly change colour at different pH, as previously described [26].

Cell cultures

AGS cells were grown at 37°C in DMEM F12 supplemented with 100 units penicillin/mL, 100 mg streptomycin/mL, L-glutamine 2 mM, and 10% heat-inactivated FBS (Euroclone S.p.A, Pero, Italy), in a humidified atmosphere containing 5% CO_2.

Caco-2 cells were cultured in DMEM containing 10 % FBS, 4 mM L-glutamine, 1 mM sodium

pyruvate, 1% non essential amino acids, and 100 units penicillin/mL, 100 mg streptomycin/mL. The cells were incubated at 37°C in humidified atmosphere with 5% CO_2 until 50% of confluence, and medium was changed every other day.

Secreted IL-8 measurement

Cells were grown in 24-well plates for 48 hrs (30000 cells/well) before the cytokine treatment. IL-8 was quantified using Human Interleukin-8 ELISA Development Kit as described below. Briefly, Corning 96 well EIA/RIA plates from Sigma-Aldrich (Milan, Italy), were coated with the antibody provided in the ELISA Kit (Peprotech Inc., London, UK), overnight at 4°C. After blocking the reaction, 200 µl of samples in duplicate, were transferred into wells at room temperature for 1 hr. The amount of IL-8 in the samples was detected by spectroscopy (signal read: 450 nm, 0.1 s) by the use of biotinylated and streptavidin-HRP conjugate antibodies, evaluating 3,5,3,59-tetramethylbenzidine (TMB) substrate reaction. Quantification of IL-8 was done using an optimized standard curve supplied with the ELISA Kit (8.0–1000.0 pg/mL). The IL-8 secretion (induced by TNF-α or IL-1β, both at 10 ng/mL) was tested after 6 hrs treatment in the presence of undigested or digested extract (10-100 µg/mL). EGCG (20 µM) was used as reference inhibitor of IL-8 secretion (see figure legend).

Transient transfection assay

To evaluate the NF-κB driven transcription and IL-8 promoter activity, AGS cells were plated in 24-well plates (30000 cells/well); after 48 hours, cells were transfected by calcium-phosphate method with 100 ng/well of native IL-8-LUC or 50 ng/well NF-κB-LUC, a plasmid containing the luciferase reporter gene under the control of NF-κB responsive promoter. After 16 hours, cells were placed in a medium FBS-free, and challenged with TNF-α or IL-1β at 10 ng/mL in the presence of VVWE (5–100 µg/mL), before and after *in vitro* digestion. Six hours after, cells were harvested and luciferase assay was performed using Britelite™ Plus reagent (PerkinElmer Inc. Massachusetts, USA) according to manufacturer's instructions. Data were expressed considering 100% the luciferase activity related to the cytokine-induced promoter activity.

Caco-2 cells were plated at the density of 4 x 10^4 cells/mL on 24-wells plates. 48 hours after, cells were transiently transfected with the plasmids NF-κB-LUC or native IL-8-LUC, both at 500 ng/well, using Lipofectamine 2000 (Life Technologies, Monza, Italy) in the ratio 2 μl reagent : 1 μg DNA, according to manufacturer's instructions. The native IL-8-LUC plasmid used in these experiments contains sequences responsive to several transcription factors such as activator protein 1 (AP-1), CCAAT-enhancer-binding protein-β (C/EBPβ), and NF-κB. Sixteen hours after, cells were treated for 6 hours with increasing concentrations of VVWE in the presence of the pro-inflammatory mediators (TNF-α or IL-1β, 10 ng/mL). Luciferase assay was performed as above described.

NF-κB nuclear translocation

To assess the effects of the extract on NF-κB (p65) nuclear translocation, Caco-2 cells were plated in 100 mm dishes (1.5 x 10^6 cells/dish) with fresh complete medium for 48 hrs. Then, medium was replaced with fresh medium FBS-free containing different concentrations of extracts in the presence of pro-inflammatory cytokines (10 ng/mL) for 1 hr. AGS cells were plated as above described for Caco-2 cells. After 48 hrs, cells were treated with TNF-α and IL-1β (10 ng/mL), for 1 hr and the extract was tested at 10–50 μg/mL. Nuclear extracts were prepared using Nuclear Extraction Kit from Cayman Chemical Company (Michigan, USA) and stored at -80°C until assayed. The same quantity of total nuclear proteins, measured by the method of Bradford, was used to assess NF-κB nuclear translocation using the NF-κB (p65) transcription factor assay kit (Cayman) followed by spectroscopy (signal read 450 nm, 0.1 s). Data were expressed considering 100% the absorbance related to the cytokine-induced NF-κB nuclear translocation. EGCG (20 μM) was used as reference inhibitor of NF-κB translocation. Results are the mean ± sd of three experiments in triplicate.

Cytotoxicity assay

The integrity of the cell morphology before and after treatment was assessed by light microscope inspection. Cell viability was measured by MTT method. No sign of cytotoxicity was observed in

cells treated with VVWE at 5–500 μg/mL for 6 hrs in both cell lines used in the present study, thus indicating that the extract does not influence cell viability in the gastrointestinal tract before and after digestion.

Statistical analysis

All data are the mean ± SD of at least three experiments performed in duplicate (ELISA assays) or triplicate (transfection assays). Data were analyzed by unpaired one-way analysis of variance (ANOVA), or two-way analysis of variance (ANOVA) followed by Bonferroni as post-hoc test. Statistical analyses were done using GraphPad Prism 5.02 software (GraphPad Software Inc., San Diego, CA, USA). $p<0.05$(*) was considered statistically significant. IC_{50} was calculated using GraphPad Prism 5.02.

Results

Validation of HPLC method for VVWE analysis

HPLC-DAD method to quantify the main anthocyanins (delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside) and the other flavonoids (rutin, hyperoside, quercetin-3-O-glucoside, quercetin-3-O-glucuronide, kaempferol-3-O-glucoside) or caffeic acid derivatives (caftaric acid) was developed and validated according to FDA guidelines. Our results show that the method is precise (RSD% < 15%), sensitive and capable of separating the compounds occurring in *VVWE* (**Table 1**).

Phytochemical characterization of *Vitis vinifera* L. water extract

The total phenols content in the extract was 146.3±4.2 mg GAE/g whereas anthocyanins were and 4.3±0.01 mg/g expressed as cyanidin-3-glucoside equivalents. These results are in agreement with previous data from scientific literature [27]. The minimum content of total polyphenols and anthocyanins in *Vitis vinifera* L. leaves, as required by the *French Pharmacopoeia* (10th Edition) is 4.0 % and 0.20%, respectively [28]. Total flavonoid content was 87.09 mg/g, according to data

reported in the literature [29] [30] [27]. Since colorimetric methods typically lack specificity, the results were compared to data obtained by HPLC-DAD analysis.

Figure 1 and **2** show HPLC-DAD analysis of anthocyanins, flavonols, and caffeic acid derivatives **(A)** before *in vitro* digestion; **(B)** after *in vitro* gastric digestion; **(C)** after *in vitro* gastro-intestinal digestion. Anthocyanins abundance in VVWE was as follows: cyanidin-3-O-glucoside > peonidin-3-O-glucoside > malvidin-3-O-glucoside > petunidin-3-O-glucoside (**Table 2**). As regards flavonols, quercetin-3-O-glucuronide was the most abundant. Among caffeic acid derivatives, caftaric acid was present in the extract whereas chlorogenic, caffeic and ferulic acids were undetectable (values < LOD, as reported in **Table 2**).

Gastric digestion generally led to a mild reduction of the compounds concentration, more pronounced for flavonols (ranging from -4.19 to -23.48%) than for anthocyanins (ranging from -3.93 to -13.68 %). The only caffeic acid derivative previously identified, caftaric acid, showed a slight decrease (-8.91 %). After gastro-intestinal digestion, the concentration of flavonols and anthocyanins dramatically decreased; reduction was particularly high for delphinidin-3-O-glucoside and malvidin-3-O-glucoside (around 70% reduction). Caftaric acid showed a mild reduction (-20%) with respect to flavonoids.

VVWE inhibits TNFα-induced IL-8 release and promoter activity in AGS cells

Since IL-8 is highly involved in gastritis development, contributing to exacerbate inflammation in this district, the following experiments were devoted to evaluate the effect of VVWE on IL-8 secretion and promoter activity induced by TNFα in human epithelial gastric AGS cells. The water extract inhibited TNFα-induced IL-8 secretion and promoter activity in a concentration dependent manner (**Figure 3 A, B,** white columns). The effects on the IL-8 promoter activity were more pronounced than those observed on IL-8 release (IC_{50}: 9.51 and 56.85 μg/mL, respectively).

To investigate if the effect observed on IL-8 released by gastric epithelial cells could be maintained at the acidic conditions of the stomach, we subjected VVWE to *in vitro* gastric digestion. Results

are shown in **Figures 3 A, B,** black columns). *In vitro* digestion led to a significant decrease of the anti-inflammatory activity elicited by VVWE in AGS cells; in particular, IC_{50} on IL-8 secretion and promoter activity were respectively two and five folds higher than those observed before *in vitro* gastric digestion (**Table 3**), thus suggesting that some biologically active molecules occurring in the extract could not be stable in the acidic conditions of the stomach.

To gain molecular insights we tested VVWE on the NF-κB driven transcription and nuclear translocation; in fact, it is widely reported in the literature that IL-8 expression is dependent by NF-κB. AGS cells were treated with increasing concentrations of the extract (5-100 μg/mL), and challenged with TNFα. The effect was evaluated treating cells with the extract and the stimulus for one (nuclear translocation) or six hours (transcription). Our results clearly show that VVWE inhibits the NF-κB pathway by interfering with the NF-κB driven transcription and nuclear translocation (IC_{50}: 17.01 and 23.55 μg/mL, respectively). According to results obtained on IL-8 secretion and promoter activity, gastric digestion decreased the ability of the extract to impair NF-κB nuclear translocation (IC_{50}: 74.25 μg/mL) whereas no statistically significant changes in the IC_{50} were observed on the NF-κB driven transcription (IC_{50}: 21.65 μg/mL) (**Figure 4** panels **A, B** and **Table 3**).

VVWE shows mild effects on cytokine-induced IL-8 release and promoter activity in Caco-2 cells

In order to assess whether the effect elicited by the extract could be maintained in the gut, we performed additional *in vitro* experiments in undifferentiated Caco-2 cells, a widely used cell model of human colonocytes [31]. TNFα was not able to induce IL-8 secretion in Caco-2 cells but stimulated IL-8 promoter activity (**Table 3**). As shown in **Figure 5**, VVWE inhibited TNFα-induced IL-8 promoter activity in a concentration-dependent fashion (IC_{50}: 2.41 μg/mL); VVWE inhibited the NF-κB pathway as well by interfering with the NF-κB driven transcription and nuclear translocation induced by TNFα (IC_{50}: 2.65 and 32.49 μg/mL, respectively); *in vitro* digestion

almost completely abolished the effect of VVWE, indicating a further loss of activity during passage from the stomach into the gut (**Figure 7**, panels **A**, **B** and **Table 3**).

IL-1β induced IL-8 secretion and promoter activity in Caco-2 cells; VVWE was able to inhibit IL-8 secretion although at higher concentration hardly reachable in the gut (IC_{50}: 96.1 μg/mL) (**Figure 6**, panel **A**) whereas no effect was found on the promoter (**Figure 6**, panel **B**), thus indicating that inhibition of IL-8 release by VVWE involves mechanisms other than promoter activity. The effect on the IL-8 secretion was parallel to a reduction of the NF-κB impairment (**Figure 7**, panel **C**, **D** and **Table 3**). Moreover, after *in vitro* gastro-intestinal digestion, the effect on IL-8 expression occurred only at 100 μg/ml and the effect on the NF-κB pathway was completely lost (**Figure 6** and **7**, black columns). Quantitative analysis performed after *in vitro* intestinal digestion confirmed a high decrease of flavonoids ranging from -26.72% (rutin) to -71.58% (delphinidin-3-O-glucoside) (**Table 2**).

Discussion

An increasing number of evidences support for the health beneficial effects of botanicals against gastro-intestinal inflammation, including PFS and dietary polyphenols. Among ingredients of PFS, red vine leaves are widely used mainly in association with other botanicals; however, no studies regarding the anti-inflammatory activity in the gastrointestinal tract have been reported so far.

Our results show that the water extract from red vine leaves inhibits TNFα-induced IL-8 secretion and expression in human gastric epithelial cells; the effect should be maintained, although to a lesser extent, after gastric digestion. On the contrary, the effect after intestinal digestion is dramatically decreased since degradation of the active components in the gut does not allow the extract to efficiently counteract TNFα or IL-1β induced IL-8 expression and NF-κB pathway. The main molecular target of VVWE at the gastric level includes TNFα-induced activation of NF-κB and occurs at concentrations easily reachable after PFS consumption based on red vine leaves water

extract as ingredient.

According to the literature, the compounds responsible for the biological activity of vine are flavonoids, in particular flavonols and anthocyanins. Evidences gathered in the last decade have clearly demonstrated that pH of the stomach (1-2) ensures that anthocyanins are maintained as the flavylium cation, which is their most stable form; the stability of these molecules under the gastric conditions has been confirmed by several studies [32] [33]. Conversely, the neutral pH of the intestine and the presence of human microflora convert anthocyanins into a variety of metabolites, leading to a rapid degradation to their corresponding phenolic acids and aldehydes through cleavage of the C ring [34] [35, 36]. Our data are in agreement with the literature since after gastro-intestinal digestion we observed a strong decrease in anthocyanin content, ranging from -38% to over -71%. Unfortunately, information regarding stability in the gastro-intestinal tract of compounds occurring in vine leaves other than flavonoids lack in the literature.

Results reported herein suggest that stomach could be preferentially the target organ for the anti-inflammatory activity elicited by red vine extract. HPLC-DAD analysis revealed that anthocyanins are present in the extract, after gastric digestion, at sub-micromolar concentrations (around 150-500 nM). Anthocyanins from black soybean have been shown to inhibit *H. pylori*-induced inflammation in gastric AGS cells, acting on IL-8 production at concentrations dramatically higher (close to 100 μM) with respect to those present in our extract, thus suggesting that their contribution as anti-inflammatory ingredients in the extract is probably negligible [37]. On the contrary, quercetin-3-O-glucuronide and quercetin 3-O-glucoside are the most abundant flavonols after *in vitro* gastric digestion, reaching each around 2% of the whole content of the extract. Previous studies have demonstrated that quercetin glycosides are stable in the human stomach and small intestine [38], suggesting that these compounds could reach micromolar concentrations in this site after consumption of water extracts from red vine leaves. Moreover, quercetin 3-O-glucuronide was found to inhibit indomethacin-induced gastric damage in rats [39]. Flavonoids, including quercetin derivatives, are well-known inhibitors of the NF-κB pathway [40] [41].

Taken together, these considerations suggest that quercetin glycosides more than anthocyanins could be responsible for the anti-inflammatory activity elicited at the gastric level whereas their reduction could lead to the loss of the biological activity in the gut. However, the contribution of other compounds to the anti-inflammatory activity cannot be excluded.

Conclusions

Our results show that the water extract from red vine leaves inhibits TNFα-induced IL-8 secretion and expression in human gastric epithelial cells, and the effect should be maintained, although to a lesser extent, after gastric digestion. Our findings suggest that PFS containing water extracts from *Vitis vinifera* L. leaves could be useful to inhibit/attenuate gastric inflammation inhibiting IL-8 secretion and expression through impairment of the NF-κB pathway.

ABBREVIATIONS USED

Dulbecco's Modified Eagle's Medium (DMEM); Plant Food Supplement (PFS); inflammatory bowel diseases (IBD); European Scientific Cooperative on Phytotherapy (ESCOP); *Vitis vinifera* L. water extract (VVWE); Diode Array Detector (DAD); fetal calf serum (FBS); Epigallocatechin gallate (EGCG); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); 3,5,3,59-tetramethylbenzidine (TMB).

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FIGURE CAPTIONS

Figure 1: Chromatographic profile of anthocyanins in VVWE: **(A)** before *in vitro* digestion; **(B)** after *in vitro* gastric digestion; **(C)** after *in vitro* gastro-intestinal digestion. **1**: delphinidin-3-O-glucoside; **2**: cyanidin-3-O-glucoside; **3**: petunidin-3-O-glucoside; **4**: peonidin-3-O-glucoside; **5**: malvidin-3-O-glucoside.

Figure 2: Chromatographic profile of flavonols and caffeic acid derivatives in VVWE: **(A)** before *in vitro* digestion; **(B)** after *in vitro* gastric digestion; **(C)** after *in vitro* gastro-intestinal digestion. **6**: caftaric acid; **7**: rutin; **8**: hyperoside; **9**: quercetin-3-O-glucoside; **10**: quercetin-3-O-glucuronide; **11**: kaempferol-3-O-glucoside.

Figure 3: Effect of VVWE on the TNFα-induced IL-8 secretion (panel **A**) and promoter activity (panel **B**) before (white columns) and after (black columns) *in vitro* gastric digestion. AGS cells were treated for 6 hrs with TNFα (10 ng/mL) and VVWE (5-100 μg/mL). Secreted IL-8 was evaluated by ELISA assay whereas IL-8 promoter activity in transiently transfected AGS cells by luciferase assay. Basal (without TNFα) and control (with TNFα) levels of IL-8 were 8.5 and 217.8 pg/ml, respectively. The graphs show the means ± SD of at least 3 experiments performed in duplicates or triplicates. Statistical analysis: one-way ANOVA, followed by Bonferroni as post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. TNFα alone. EGCG 20 μM was used as reference inhibitor of IL-8 secretion (70.9 % inhibition) and IL-8 promoter activity (81.9 % inhibition); the effect of the reference inhibitor is in agreement to that reported in the scientific literature.

Figure 4: Effect of VVWE on the TNFα-induced NF-κB driven transcription (panel **A**) and nuclear translocation (panel **B**) before (white columns) and after (black columns) *in vitro* gastric digestion. AGS cells were treated for 1 hr (nuclear translocation assay) or 6 hrs (NF-κB driven transcription) with TNFα (10 ng/mL) and VVWE (5-100 μg/mL). The graphs show the means ± SD of at least 3 experiments performed in duplicates or triplicates. Statistical analysis: one-way ANOVA, followed by Bonferroni as post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. TNFα alone. EGCG 20

μM was used as reference inhibitor of TNFα-induced NF-κB driven transcription (87.1 % inhibition) and NF-κB nuclear translocation (around 100% inhibition); the effect of the reference inhibitor is in agreement to that reported in the scientific literature.

Figure 5: Effect of VVWE on the TNFα-induced IL-8 promoter activity before (white columns) and after (black columns) *in vitro* gastric digestion. AGS cells were treated for 6 hrs with TNFα (10 ng/mL) and VVWE (1-100 μg/mL). IL-8 promoter activity was measured in transiently transfected AGS cells by luciferase assay. The graphs show the means ± SD of at least 3 experiments performed in triplicates. Statistical analysis: one-way ANOVA, followed by Bonferroni as post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. TNFα alone. EGCG 20 μM was used as reference inhibitor (64.2 % inhibition); the effect of the reference inhibitor is in agreement to that reported in the scientific literature.

Figure 6: Effect of VVWE on the IL-1β-induced IL-8 secretion (panel **A**) and promoter activity (panel **B**) before (white columns) and after (black columns) *in vitro* gastro-intestinal digestion. Caco-2 cells were treated for 6 hrs with IL-1β (10 ng/mL) and VVWE (10-100 μg/mL). Secreted IL-8 was evaluated by ELISA assay whereas IL-8 promoter activity in transiently transfected Caco-2 cells by luciferase assay. Basal (without IL-1β) and control (with IL-1β) levels of IL-8 were 1.2 and 68.0 pg/ml, respectively. The graphs show the means ± SD of at least 3 experiments performed in duplicates or triplicates. Statistical analysis: one-way ANOVA, followed by Bonferroni as post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. IL-1β alone. EGCG 20 μM was used as reference inhibitor of IL-8 promoter and secretion (around 65 % inhibition); the effect of the reference inhibitor is in agreement to that reported in the scientific literature.

Figure 7: Effect of VVWE on the NF-κB driven transcription induced by TNFα (panel **A**) or IL-1β (panel **C**) and nuclear translocation (panel **B** and **D**) before (white columns) and after (black columns) *in vitro* gastro-intestinal digestion. Caco-2 cells were treated for 1 hr (nuclear

translocation assay) or 6 hrs (NF-κB driven transcription) with TNFα or IL-1β (10 ng/mL) and VVWE. The graphs show the means ± SD of at least 3 experiments performed in duplicates or triplicates. Statistical analysis: one-way ANOVA, followed by Bonferroni as post-hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. TNFα or IL-1β alone. EGCG 20 μM was used as reference inhibitor of TNFα or IL-1β-induced NF-κB driven transcription (58.7 and 12.0 % inhibition, respectively) and NF-κB nuclear translocation (45.9 and 19.0 % inhibition, respectively); the effect of the reference inhibitor is in agreement to that reported in the scientific literature.

Table 1: HPLC-DAD validation for analysis of flavonols, caffeic acid derivatives and anthocyanins.

Compound	Precision*		Linearity		LOD (ng/mL) (mean±SD)	LOQ (ng/mL) (mean±SD)
	Intraday (CV%)	Interday (CV%)	Linear Range (µg/mL)	R^2		
Caftaric acid	6.92	5.07	2.5-50	0.993	43.5±2.7	145.1±8.9
Rutin	3.84	3.14	0.1-2	0.996	4.3±0.3	14.3±1.0
Hyperoside	6.27	4.09	0.1-5	0.998	3.3±0.5	11.0±1.4
Quercetin-3-O-glucoside	5.89	3.74	2.5-25	0.998	3.5±0.5	11.7±1.5
Quercetin-3-O-glucuronide	7.60	6.05	0.25-10	0.998	4.9±0.7	16.4±2.1
Kaempferol-3-O-glucoside	6.22	4.07	0.1-5	0.998	4.0±0.5	13.1±1.7
Delphinidin 3-O-glucoside	2.00	8.19	0.1-10.0	0.995	1.8±0.2	5.9±0.6
Cyanidin 3-O-glucoside	3.46	5.21	0.1-10.0	0.992	1.7±0.1	5.6±0.4
Petunidin 3-O-glucoside	3.79	6.65	0.1-10.0	0.994	2.6±0.2	8.6±0.6
Peonidin 3-O-glucoside	4.80	4.44	0.06-10.0	0.996	1.7±0.1	5.9±0.4
Malvidin 3-O-glucoside	5.05	5.29	0.2-12.5	0.994	2.3±0.1	7.8±0.4

*Results are the mean of three concentrations

Table 2: VVWE content before and after *in vitro* gastric or gastrointestinal digestion

	Before gastric digestion (mg/g)*	After gastric digestion (mg/g)*	After gastro-intestinal digestion (mg/g)*
Caffeic acid derivatives			
Caftaric acid	9.99±0.35	9.10±0.64 (-8.91)	7.99±0.16 (-20.02)
Caffeic acid	n.d.^	n.d.^	n.d.^
Chlorogenic acid	n.d.§	n.d.§	n.d.§
Ferulic acid	n.d.°	n.d.°	n.d.°
Flavonols			
Rutin	1.31±0.05	1.15±0.06 (-12.21)	0.96±0.02 (-26.72)
Hyperoside	2.30±0.17	1.76±0.13 (-23.48)	1.31±0.02 (-43.04)
Quercetin 3-O-glucoside	21.68±0.91	17.92±0.65 (-17.34)	13.40±0.28 (-38.19)
Quercetin 3-O-glucuronide	29.14±1.92	27.92±3.10 (-4.19)	17.41±0.06 (-40.25)
Kaempferol 3-O-glucoside	3.77±0.06	3.03±0.11 (-19.63)	2.31±0.04 (-38.73)
Anthocyanins			
Delphinidin 3-O-glucoside	0.95±0.03	0.82±0.03 (-13.68)	0.27±0.01 (-71.58)
Cyanidin 3-O-glucoside	2.29±0.04	2.20±0.05 (-3.93)	1.42±0.02 (-37.99)
Petunidin 3-O-glucoside	0.66±0.05	0.61±0.01 (-7.58)	0.21±0.01 (-68.19)
Peonidin 3-O-glucoside	1.91±0.06	1.66±0.02 (-13.09)	0.94±0.04 (-50.78)
Malvidin 3-O-glucoside	1.27±0.07	1.10±0.04 (-13.39)	0.37±0.03 (-70.87)

*Data are expressed as mg of pure compound/g VVWE (mean ± SD). In brackets are reported the percentage of pure compounds reduction after *in vitro* gastric or gastrointestinal digestion.

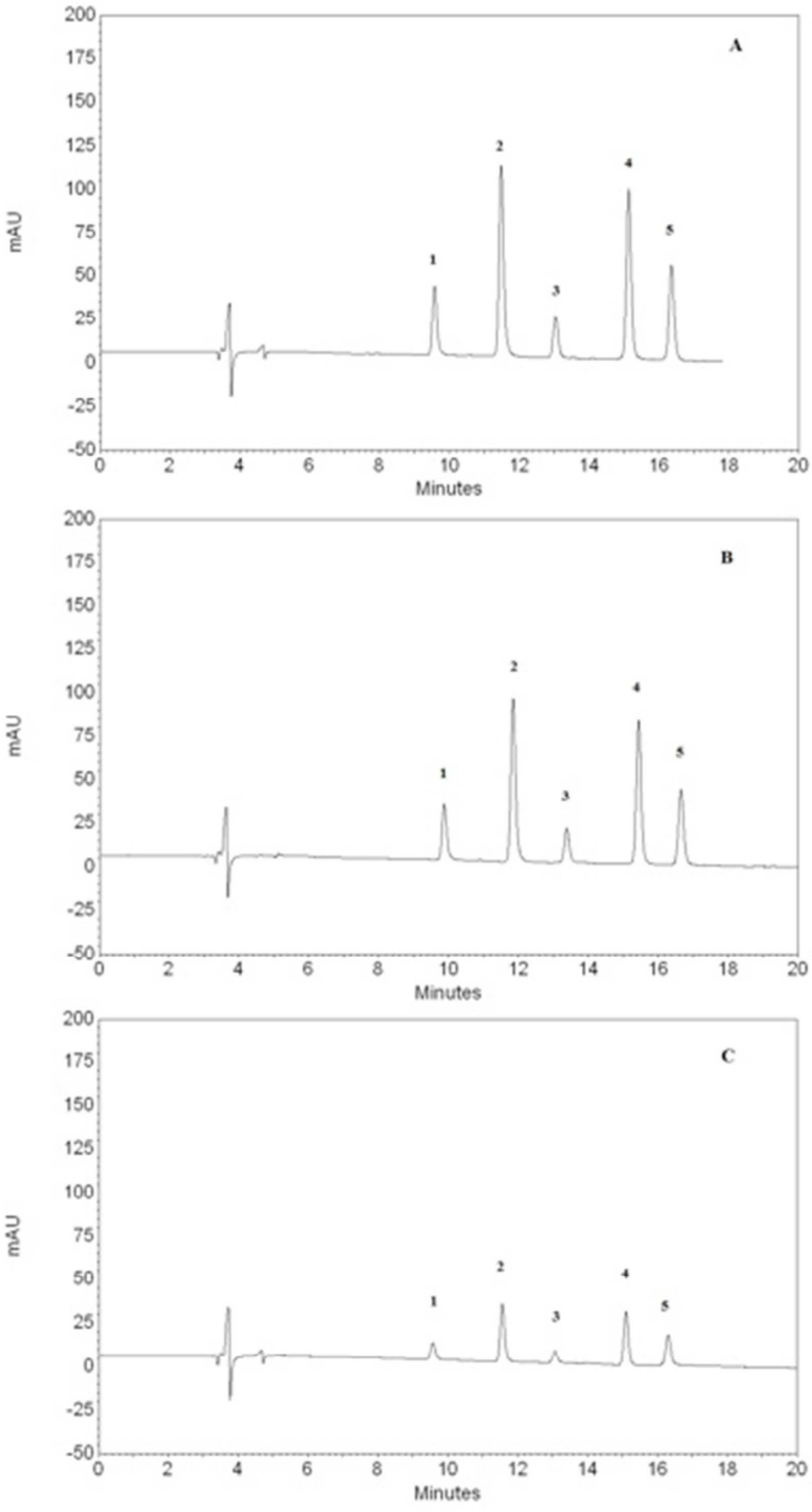
n.d.: not detectable. ^ < LOD (14.7 ng/mL); § < LOD (22.0 ng/mL); °< LOD (16.0 ng/mL)

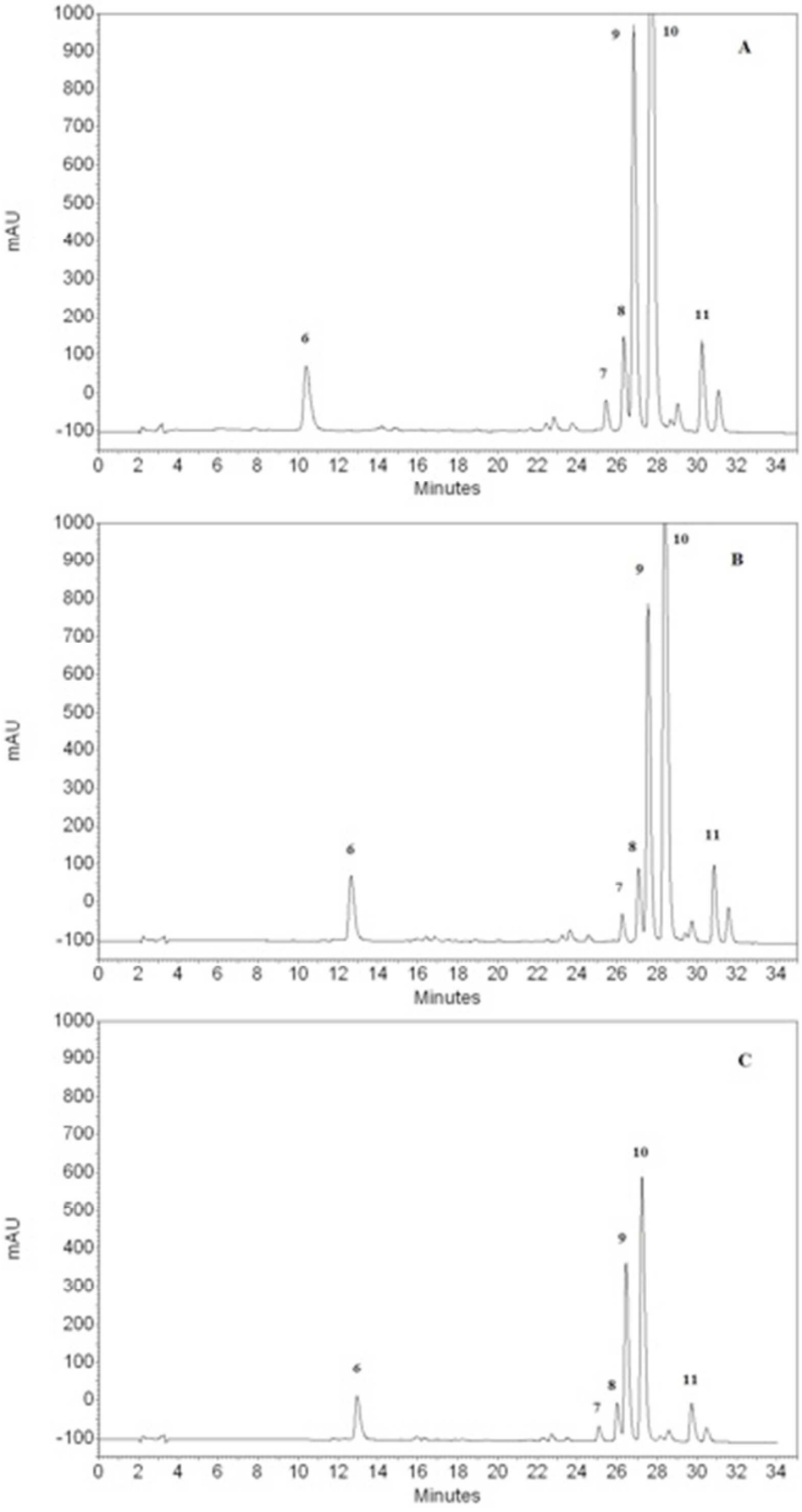
Table 3: IC_{50} of VVWE against IL-8 secretion/promoter activity and NF-κB pathway in human epithelial gastric (AGS) and intestinal (Caco-2) cells

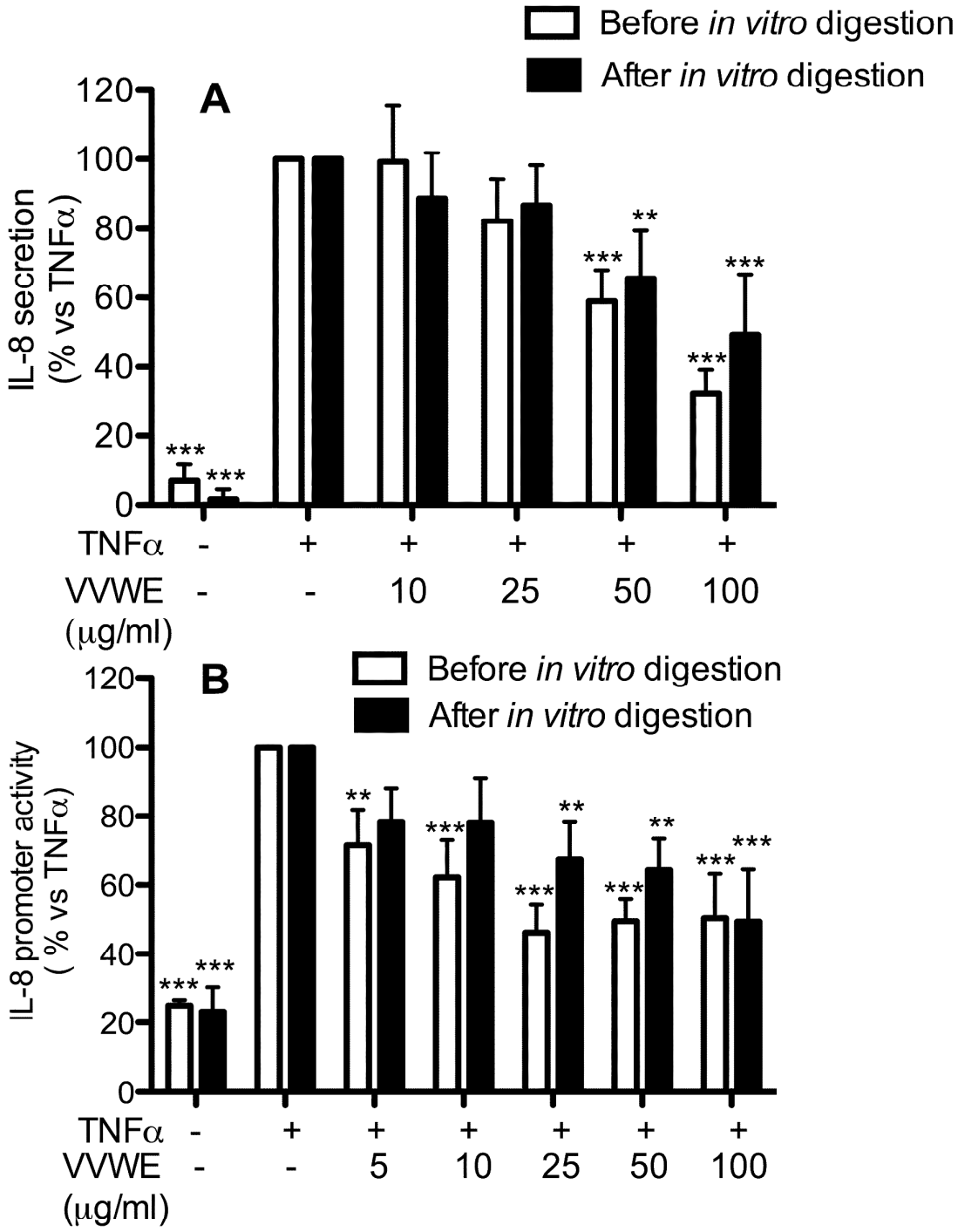
AGS cells	**Before *in vitro* gastric digestion**	**After *in vitro* gastric digestion**
	IC_{50} µg/ml (mean ± SD)	
	TNFα	
IL-8 secretion	56.85 ± 5.2	97.91 ± 21.9
IL-8 promoter activity	9.51 ± 3.9	43.44 ± 14.6
NF-κB driven transcription	17.01 ± 2.0	21.65 ± 3.9
NF-κB nuclear translocation	23.55 ± 3.9	74.25 ± 3.9

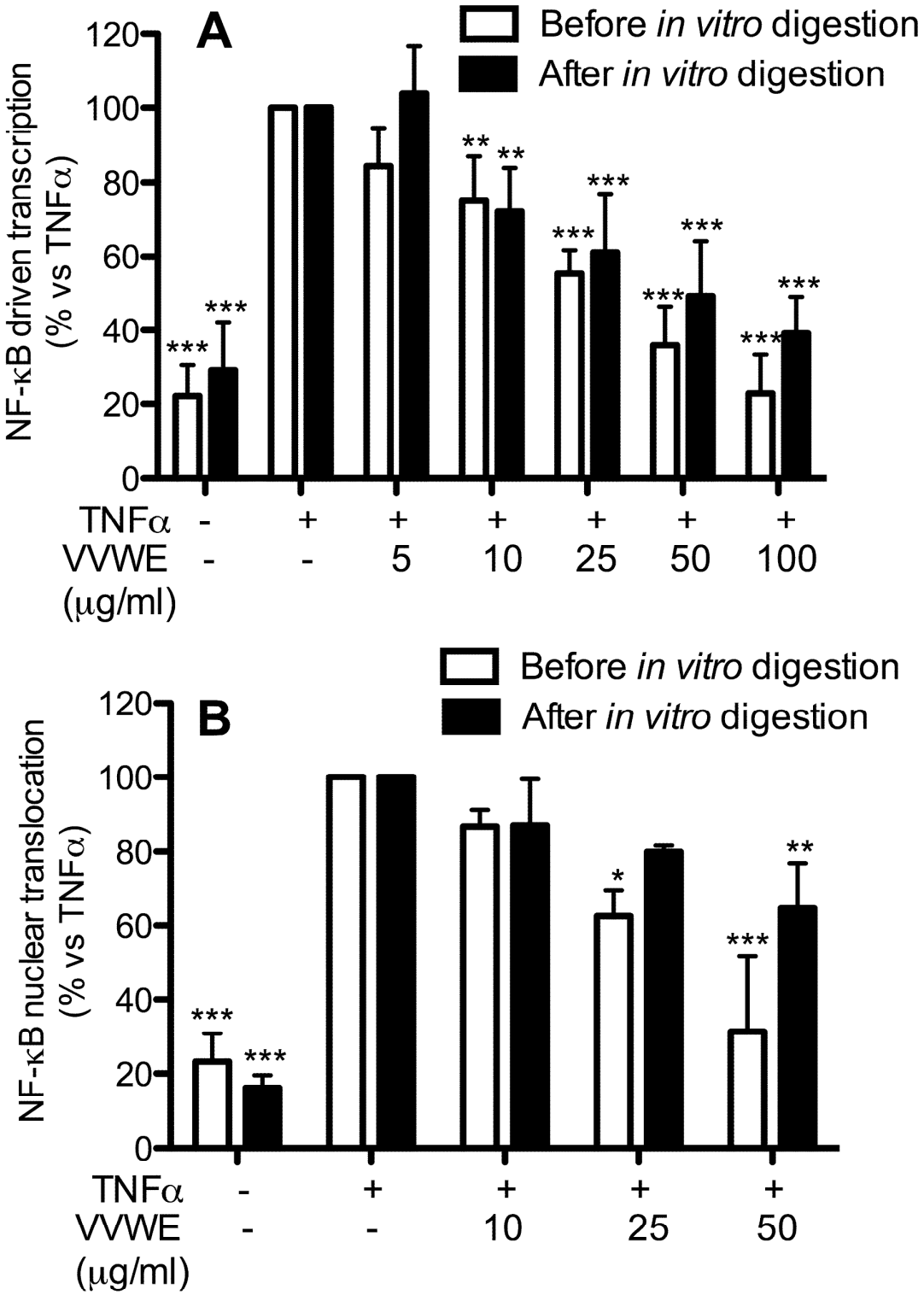
Caco-2 cells	**Before *in vitro* gastro-intestinal digestion**		**After *in vitro* gastro-intestinal digestion**	
	IC_{50} µg/ml (mean ± SD)			
	TNFα	**IL-1β**	**TNFα**	**IL-1β**
IL-8 secretion	-*	96.1 ± 18.8	-*	>50 µg/ml
IL-8 promoter activity	2.41 ± 1.1	>50 µg/ml	>50 µg/ml	>50 µg/ml
NF-κB driven transcription	2.65 ± 0.5	>50 µg/ml	>50 µg/ml	>50 µg/ml
NF-κB nuclear translocation	32.49 ± 5.6	43.5 ± 4.6	>50 µg/ml	>50 µg/ml

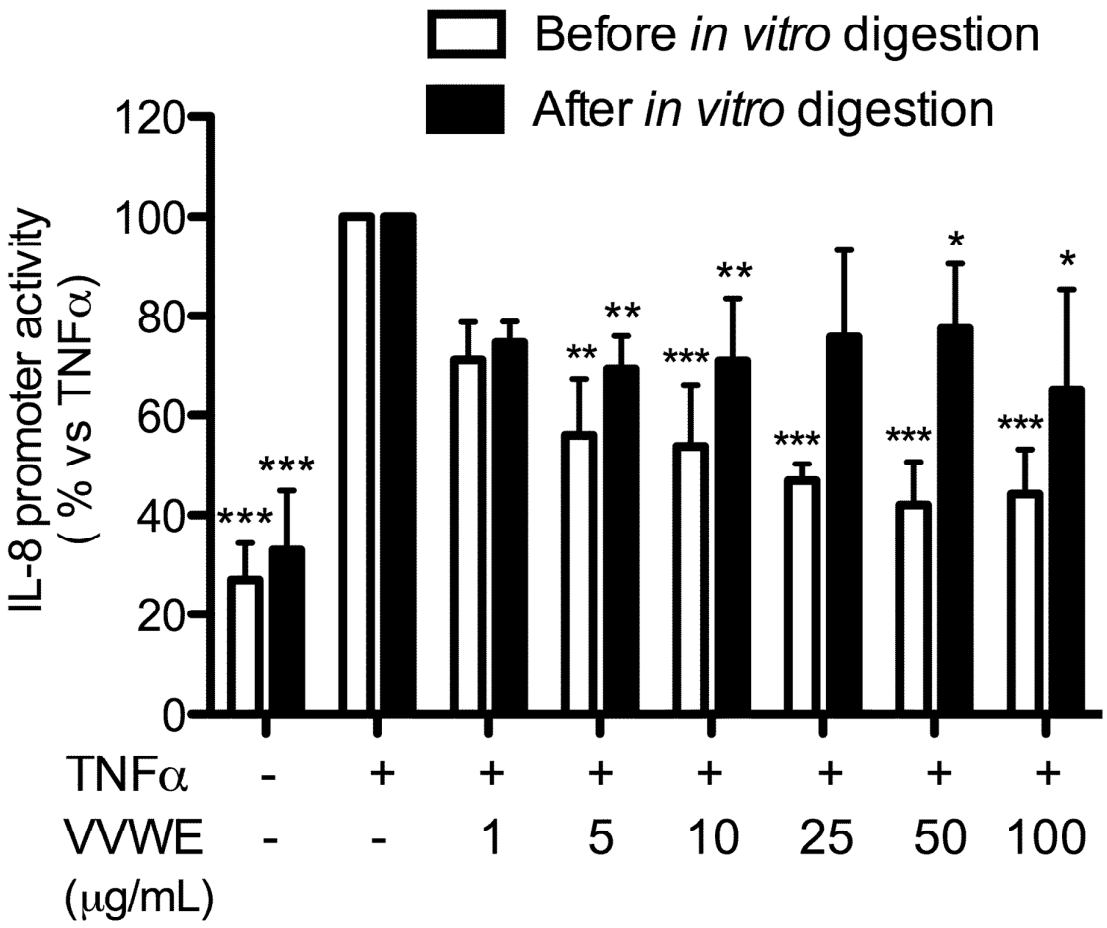
IC_{50} (µg/ml) are expressed as mean ± SD of at least three experiments performed in duplicate or triplicate. *The effect of VVWE on TNFα-induced IL-8 secretion was not tested since TNFα was not able to induce secretion of this cytokine in Caco-2 cells.

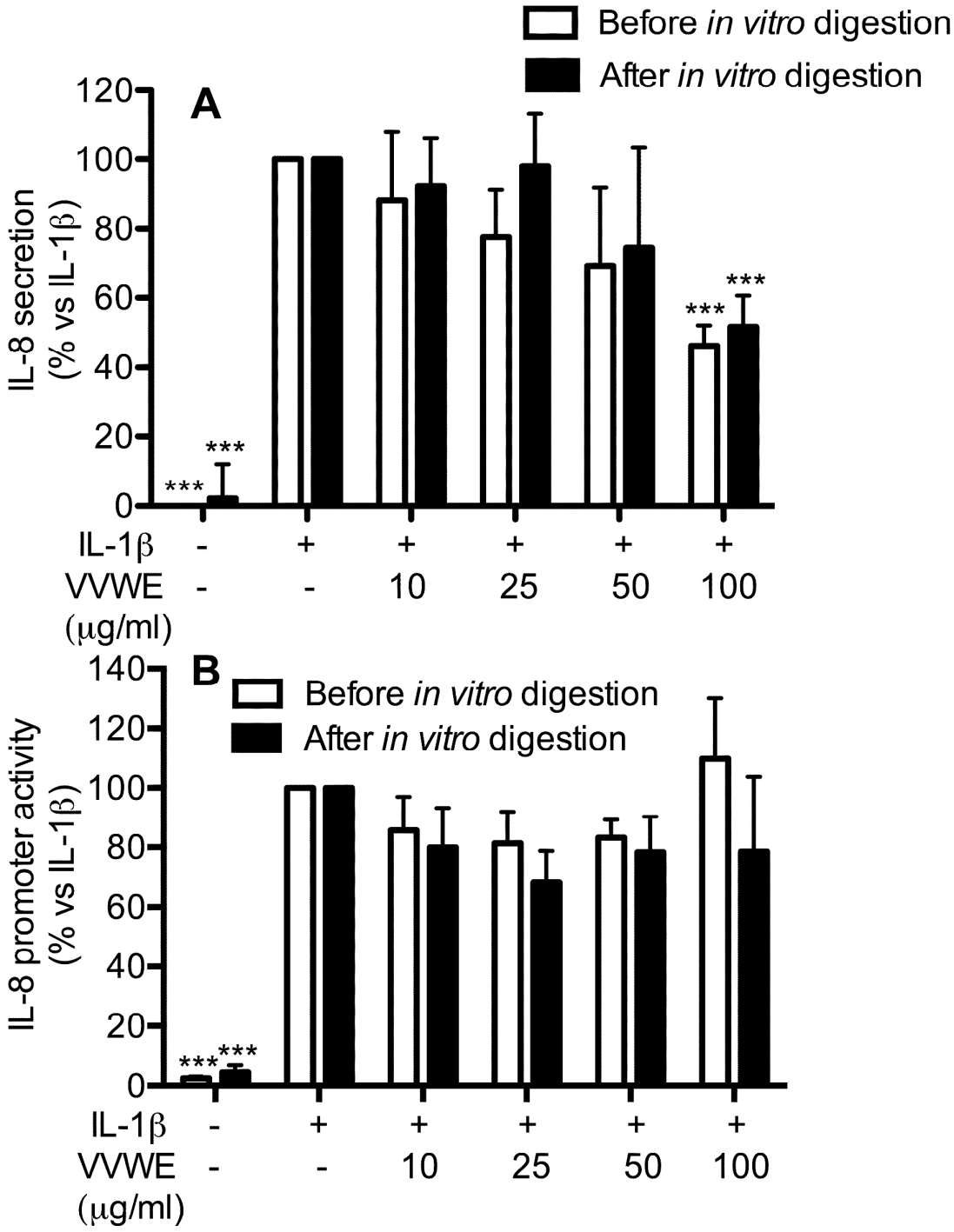


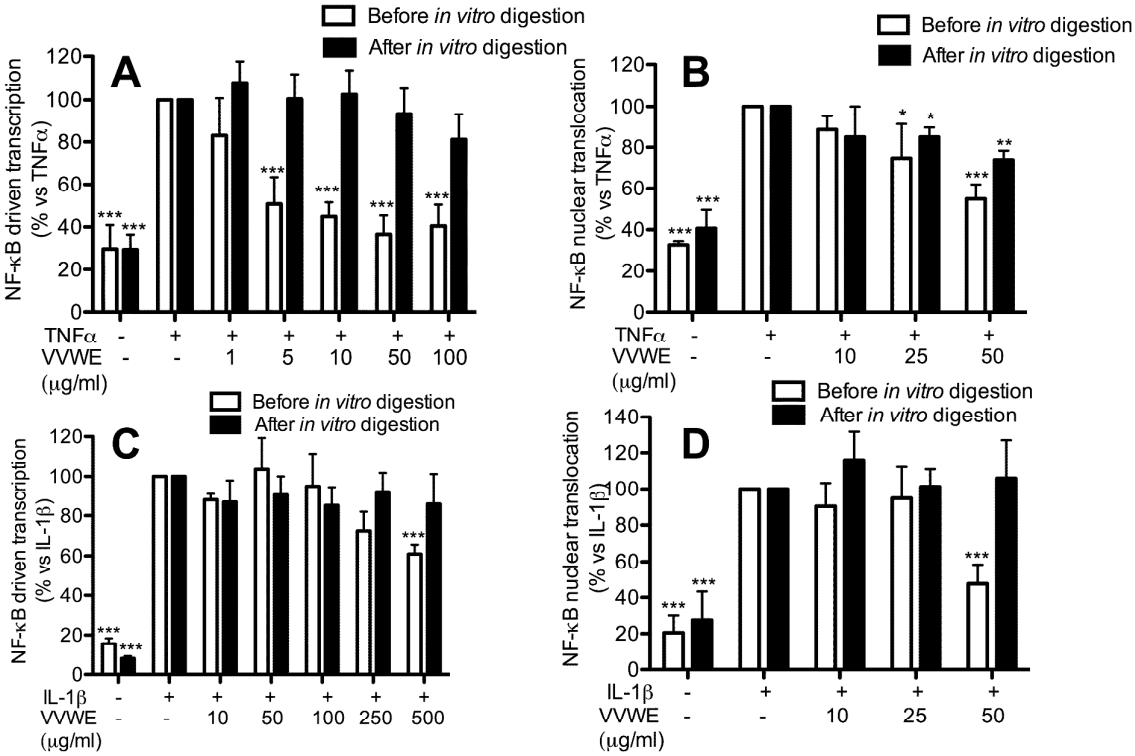


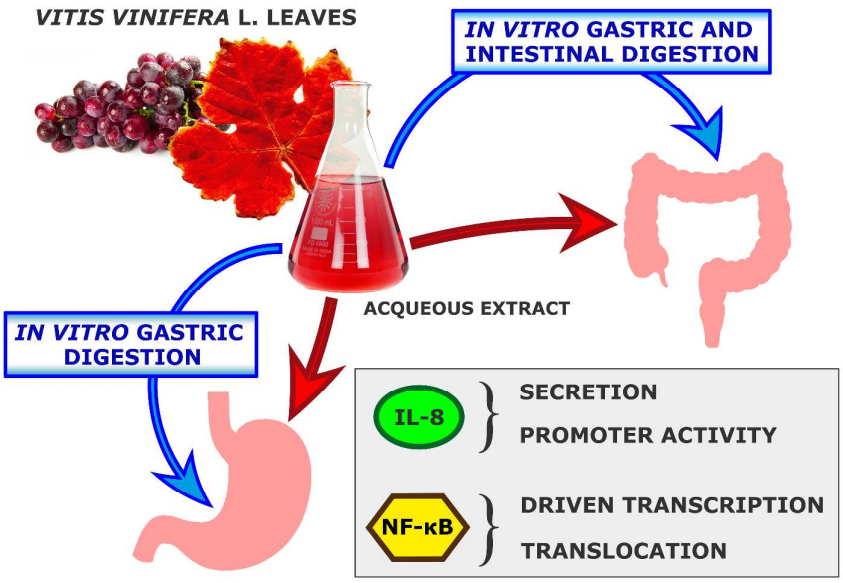












Vitis vinifera L. water extract from dried leaves maintains the anti-inflammatory activity after *in vitro* gastric digestion