

**A bio-guided assessment of the anti-inflammatory activity of hop extracts (*Humulus lupulus* L. cv. Cascade) in human gastric epithelial cells**

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## ABSTRACT

The present work aims to characterize and investigate the anti-inflammatory activity of hop extracts (cv. Cascade) in an *in vitro* model of gastric inflammation. The biological activities of hydroalcoholic and aqueous extracts from cones were evaluated by comparing IL-8 inhibition induced by TNF $\alpha$ . The hydroalcoholic extract demonstrated a higher inhibitory effect, which was just slightly affected by an *in vitro* simulated gastric digestion. The identification of active compounds was performed by a bio-guided fractionation which afforded 11 fractions, one of which inhibited IL-8 release in a concentration-dependent fashion in human gastric epithelial AGS cells. Phytochemical analysis revealed xanthohumol A and xanthohumol D as the main active components. The present study provides some experimental evidences that *Humulus lupulus* L. may exert an anti-inflammatory activity on the gastric district by the inhibition of the IL-8 secretion, partially due to its prenylated chalcones content.

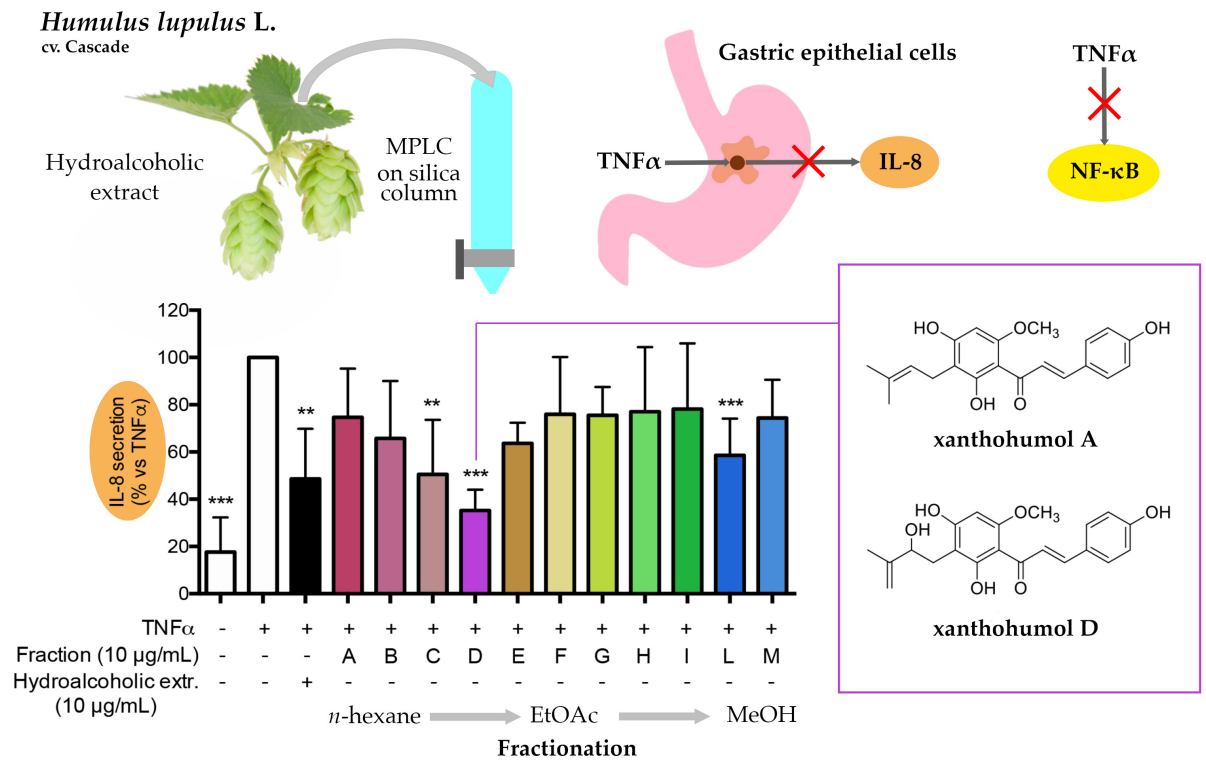
## Keywords

Hop, *Humulus lupulus* L., Gastric inflammation, IL-8, Xanthohumol, AGS cells.

## Highlights

- The hydroalcoholic extract from hop cones inhibits IL-8 release and NF- $\kappa$ B pathway.
- *In vitro* gastric digestion of hop extract slightly alters biological activity.
- Chalcones may act as major contributors to the anti-inflammatory activity.
- First demonstration of the anti-inflammatory activity of xanthohumol D in AGS cells.

## Graphical abstract

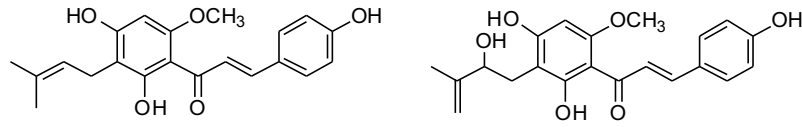


## 1. Introduction

Chronic gastritis is a common disease which represents an important background for the development of several gastric pathologies. Some of the most serious consequences of a prolonged inflammation at gastric level are peptic ulcers (Chuah et al., 2014) and cancer (Chen et al., 2016; Ohata et al., 2004). Despite a decline in the incidence in the past decades, stomach cancer has been estimated in more than 1,000,000 new cases and responsible for 783,000 deaths worldwide just in 2018 (Bray et al., 2018). *Helicobacter pylori* infection represents a major risk factor in this context (Chen et al., 2016), since this Gram negative bacterium colonizes the gastric mucosa of over 50% of the world human population (Rugge et al., 2011) and induces a persistent immune response in the host (Crabtree, 1996), which is characterized by the release of several inflammatory cytokines in the gastric mucosa, including IL-8 and TNF $\alpha$  (Crabtree, Shallcross, Heatley, & Wyatt, 1991; Gionchetti et al., 1994; Moss, Legon, Davies, & Calam, 1994; Noach et al., 1994). When exposed to TNF $\alpha$ , gastric epithelial cells release IL-8, a potent chemokine promoting neutrophil infiltration (Crabtree et al., 1994; Sharma, Tummuru, Miller, & Blaser, 1995; K Yasumoto et al., 1992), through activation of NF- $\kappa$ B (Choi et al., 2006; K Yasumoto et al., 1992), a transcription factor deeply involved in the inflammatory processes (Barkett & Gilmore, 1999; Bonizzi & Karin, 2004; Shishodia & Aggarwal, 2004). IL-8 levels in the gastric mucosa has been positively associated with the severity of gastritis (Xuan et al., 2005) and the serum levels of this chemokine correlate with the degree of gastric chronic inflammation and neutrophil infiltration (Siregar, Halim, & Sitepu, 2015), making this molecule an ideal marker to evaluate the gastric inflammatory state. Since IL-8 contributes to the amplification of the inflammation, the search for new extracts or natural leads able to inhibit this chemokine could be a strategy to prevent more severe clinical outcomes.

Hops (*Humulus lupulus* L., Cannabaceae) is a plant extensively used in human nutrition, in particular for the production of beer, in which the female inflorescences are used to provide the typical bitterness and aroma. Despite the consumption of beer is not recommended for human gastric inflammation, due to its alcohol content and relative pro-oxidant effects (de Gaetano et al., 2016), hops, as ingredients of food supplements, are considered an interesting source of bioactive compounds, such as xanthohumol A (Figure 1A), a prenylated chalcone that has shown anti-inflammatory activity in different experimental models (Cho et al., 2008; Dorn, Heilmann, & Hellerbrand, 2012; Dorn, Kraus, et al., 2010; Lee et al., 2011). The consumption of hop-based products is increasing, and hops are not only ingredients in food supplements, but also important component of non-alcoholic beverages. There is a particular interest in the research of hops active components; despite the high consumption of hops in human diet, only few studies investigated their possible beneficial effects at the gastric

level.



**Figure 1.** Chemical structures of xanthohumol A (left) and xanthohumol D (right).

The intragastric administration of a commercial hop dry extract in a rat pylorus-ligated model increased gastric juice volume without affecting acidity, a mechanism mediated by cholinergic nervous system that could positively affect the digestion process (Kurasawa, Chikaraishi, Naito, Toyoda, & Notsu, 2005). Two studies described the effect of hops against *Helicobacter pylori*. In the first study, fresh hops homogenates from three different varieties inhibited *in vitro* the growth of a clinical isolated bacterium and the effect was partly attributed to their content in  $\alpha$ - and  $\beta$ -acids (Cermak et al., 2015). In the second study, a high molecular weight fraction from hop bract extract, rich in polymerized catechins, reduced in mice the gastric damage induced by the oral administration of VacA, a vacuolating cytotoxin produced by *Helicobacter pylori* (Yahiro et al., 2005)(Yahiro et al., 2005)(Yahiro et al., 2005)(Yahiro et al., 2005). As anti-inflammatory effects, a CO<sub>2</sub> hop extract reduced the prostaglandin E<sub>2</sub> production in adenocarcinoma gastric cells (AGS) by influencing COX-2 enzyme (Tripp et al., 2005), while xanthohumol A suppressed the proliferation, apoptosis and metastasis of AGS cells via induction of ROS production and inhibition of the NF- $\kappa$ B signaling (Wei et al., 2018).

In a previous study by our group, the hop cultivar Cascade, a highly appreciated cultivar, was phytochemically characterized with the discovery of three new minor constituents, 4-hydroxycolupulone, humudifucol, and cascandone (Forino et al., 2016). In addition, xanthohumol A was found to inhibit the activity of two important pro-inflammatory enzymes, microsomal prostaglandin E<sub>2</sub> synthase (mPGES)-1 and 5-lipoxygenase (5-LO) (Forino et al., 2016).

On these bases, the present work aimed at deepening the investigation on the anti-inflammatory potential of cv. Cascade hop in gastric epithelial cells, possibly identifying the molecule(s) responsible for the effect. Attention has been paid to the extract ability to inhibit IL-8 release by gastric epithelial cells, investigating the NF- $\kappa$ B pathway as well.

## 2. Material and methods

### 2.1 General Experimental Procedures

Dulbecco's Modified Eagle's Medium/F12 (DMEM)/F12 (1:1), penicillin, streptomycin, L-glutamine and trypsin-EDTA were from Gibco (Life Technologies Italia, Monza, Italy). Foetal bovine serum (FBS), and disposable materials 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.

The reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and curcumin were from Sigma Aldrich (Milan, Italy). All reagents used for the biological assays were HPLC grade. Human TNF $\alpha$  and Human IL-8 Elisa Development Kit were from Peptotech Inc. (London, UK). All chromatographic solvents were HPLC grade or LC-MS grade for MS experiments. Acetonitrile, methanol, ethanol, formic acid, hydrochloric acid, vanillin, and iron sulfate were from Sigma Aldrich (Milan, Italy).

NMR experiments were run on Varian Unity Inova 700 spectrometer equipped with a  $^{13}\text{C}$  Enhanced HCN Cold Probe and by using a Shigemi 5 mm NMR tubes. HPLC-RI separations were performed on a Knauer (Berlin, Germany) 1800 apparatus equipped with a refractive index detector.

## *2.2 Plant material*

Hop plantlets were purchased at Garten Eickelmann (Geisenfeld, Germany) and cultivated for four years in an experimental site (Farm La Morosina, Abbiategrasso, Milan, Italy) and collected in 2014. Cones, collected at maturity, were dried at 40°C in a thermostatic room, protected from light. A voucher specimen was deposited at the Herbarium of the Botanical Garden GE Ghirardi, Department of Pharmaceutical Sciences, University of Milan, with number H1 101.

## *2.3 Preparation of the extracts*

Before extraction, hop cones were kept at 4°C to limit the chemical and enzymatic reactions. Subsequently the drug (20 g) was dried in an oven at 40°C for 24 hours and, the following day, the dried cones were pulverized using a mortar and a pestle. The obtained powder was stored inside an amber glass bottle at 4°C. To obtain the extracts, 3 grams of powder were extracted twice with 30 mL of water (aqueous extract) or 30 mL of ethanol/water 50:50 (hydroalcoholic extract) for 4 and 16 hours, respectively, at room temperature under dark conditions. The mixture was filtered through Supervalox filter paper to remove plant debris; solvents were evaporated using a Rotavapor system and extracts containing water were frozen with dry ice and alcohol and placed at -80 °C overnight, then lyophilized and maintained at -20°C. The yields (w/w) of each extraction were calculated as percentage ratio between the dried extract weight and the weight of the fresh starting material. Stock solutions of the extracts were prepared, aliquoted at 25 mg/mL and kept at -20°C; aqueous extract

was solved in a mixture of water/DMSO (50:50), while hydroalcoholic extract used an ethanol/water mixture (50:50).

#### 2.4 Fractionation of the hydroalcoholic extract

A sample of the inflorescences of *H. lupulus* cultivar Cascade (30 g), was triturated and extracted three times with 500 mL of H<sub>2</sub>O or with 500 mL of EtOH/H<sub>2</sub>O (1:1) (v/v) mixture. The extracts were combined and evaporated, and the resulting residue was separated on a MPLC (Buchi) silica gel (230–400 mesh) column (460 mm × 70 mm) eluted with a stepwise gradient of *n*-hexane to EtOAc and then to MeOH. The polarity was changed from *n*-hexane to EtOAc by increasing the EtOAc percentages by 10% (v/v), after each 250 mL, and then by EtOAc to MeOH with a 30% (v/v) increase. Following this procedure, 11 fractions were collected (A-M): A (375 mg), B (120 mg), C (68 mg), D (75 mg), E (121 mg), F (90 mg); G (77 mg), H (89 mg), I (111 mg), L (221 mg), M (300 mg). The most active fraction, fraction D (75 mg, 0.25%), was further purified on a HPLC equipped with RI detector and a Gemini 10 μm column and eluted with H<sub>2</sub>O-MeOH (2:8) to obtain pure xanthohumol A (59 mg, 0.20%) and xanthohumol D (6.8 mg, 0.023%) (Figure 1B).

#### 2.5 HPLC-DAD and LC-MS analysis

HPLC-DAD analyses of samples obtained from hydroalcoholic extraction were performed at room temperature on a Jasco PU 1580 HPLC system, equipped with MD-1510 PDA detector and Lichrocart® RP-18 column (250 x 4.6 mm, 3 μm, Merck KGaA, Darmstadt, Germany). A binary mixture composed of 0.1% formic acid in water (A) and pure acetonitrile (B) was used for elution, applying the following gradient program: 0-10 min B 10-15%, 10-45 min B 15-40%, 45-52 min B 40-100% and 52-58 min B 100%, at a flow rate of 0.6 mL/min. LC-MS analyses were performed on a Thermo Finnigan LC-MS system, equipped with a PDA detector and an LCQ Advantage (ESI+ ionization, fleet ion trap) mass spectrometer, using the same column and gradient conditions reported above. Evaluation of xanthohumol concentrations were performed by comparison with known solutions of each pure xanthohumol isomer, obtained by further purification of hop extract (Forino et al., 2016). LC-ESI-MS conditions for analysis were: Capillary Temperature 275 °C, Source Heater Temperature 150°C, Sheath Gas Flow 20.0 (Arb.Unit), Aux Gas Flow 10.0 (Arb.Unit), Source voltage 4 kV, Source Current 100 uA, Capillary Voltage 50 V.

#### 2.6 Cytotoxicity

The integrity of the cell morphology before and after treatment was assessed by light microscope inspection. Cell viability was measured, after 6 h treatment, by the 3,4,5-dimethylthiazol-2-yl-2,5-

diphenyltetrazolium bromide (MTT) method. This method evaluates the activity of a mitochondrial enzyme, which is an index of cell viability. The concentrations of the extracts which resulted cytotoxic were excluded from other biological assays.

### *2.7 Cell culture and IL-8 release measurement*

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

Cells were grown in 24-well plates for 48 h (30,000 cells per well) before the cytokine treatment. The IL-8 secretion, induced by TNF $\alpha$  at 10 ng/mL, was tested after 6 h treatment in the presence of un-digested or digested extracts (0.1–50  $\mu$ g/mL). EGCG (10  $\mu$ M) was used as the reference inhibitor (Bulgari et al., 2012; Fumagalli et al., 2016) of IL-8 secretion (50% inhibition). IL-8 was quantified using a 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  $\mu$ l of samples in duplicate were transferred into wells at room temperature for 1 h. 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 the 3,3',5,5'-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).

### *2.8 Transient transfection assay*

To evaluate the NF- $\kappa$ B driven transcription and IL-8 promoter activity, AGS cells were plated in 24-well plates (30,000 cells per well); after 48 hours, cells were transfected by the calcium-phosphate method with 100 ng per well of native IL-8-LUC or 50 ng per well of NF- $\kappa$ B-LUC, the latter containing the luciferase reporter gene under the control of a NF- $\kappa$ B responsive promoter (E-selectin promoter). The plasmid NF- $\kappa$ B-LUC was a gift of Dr. N. Marx (Department of Internal Medicine-Cardiology, University of Ulm, Ulm, Germany). After 16 h, the cells were treated with the stimulus (TNF $\alpha$  10 ng/mL) and the extract for 6 h. EGCG (10  $\mu$ M) was used as the reference inhibitor (75% inhibition for NF- $\kappa$ B-LUC driven transcription). At the end of this time, cells were lysed, and the luciferase assay was performed using the Britelite™ Plus reagent (PerkinElmer Inc., Waltham, MA, USA) according to the manufacturer's instructions. Data were expressed considering 100% of the luciferase activity related to the cytokine-induced promoter activity.



### 2.9 Evaluation of NF- $\kappa$ B nuclear translocation

For the NF- $\kappa$ B (p65) nuclear translocation assay, AGS cells were plated at the concentration of  $1.5 \times 10^6$  cells/ml in 60-mm plates. After 48 hours, cells were treated for 1 hour with the inflammatory mediator (TNF $\alpha$  10 ng/mL) and the extracts under study. Nuclear extracts were prepared using Nuclear Extraction Kit from Cayman Chemical Company (Michigan, USA) and stored at  $-80^\circ\text{C}$  until assayed. The same quantity of total nuclear proteins, measured by Bradford assay, was used to assess NF- $\kappa$ B nuclear translocation using the NF- $\kappa$ B (p65) transcription factor assay kit (Cayman), followed by spectroscopy analysis (signal read 450 nm, 0.1 s). Data were expressed considering 100% the absorbance related to the cytokine-induced NF- $\kappa$ B nuclear translocation. **EGCG (10  $\mu\text{M}$ ) was used as the reference inhibitor (85% inhibition for NF- $\kappa$ B nuclear translocation).**

### 2.10 Simulated in vitro gastric digestion

According to a well-established protocol, the gastric digestion was simulated using an in vitro approach previously described (Sangiovanni et al., 2015). Briefly, the extract (100 mg) was incubated for 5 minutes at  $37^\circ\text{C}$  with 6 mL of saliva juice, then 12 mL gastric juice were added to the suspension and the sample was incubated for 2 hours at  $37^\circ\text{C}$ . At the end of the incubation, the digested sample was centrifuged for 5 minutes at 3000 g and the supernatant frozen and lyophilized. All the samples, at different steps, were then stored at  $-20^\circ\text{C}$  until the use for biological assays.

### 2.11 Statistical Analysis

All data are expressed as mean  $\pm$  s.d.; data were analyzed by unpaired one-way analysis of variance (ANOVA) followed by Bonferroni as post-hoc test. Statistical analyses were done using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) **by comparing all the conditions with respect to the stimulated control (TNF $\alpha$ )**, considering \* $p < 0.05$ ; \*\* $p < 0.01$  and \*\*\* $p < 0.001$ . The value of  $p < 0.05$  was considered statistically significant. IC<sub>50</sub> was calculated using GraphPad Prism 5.00 software.

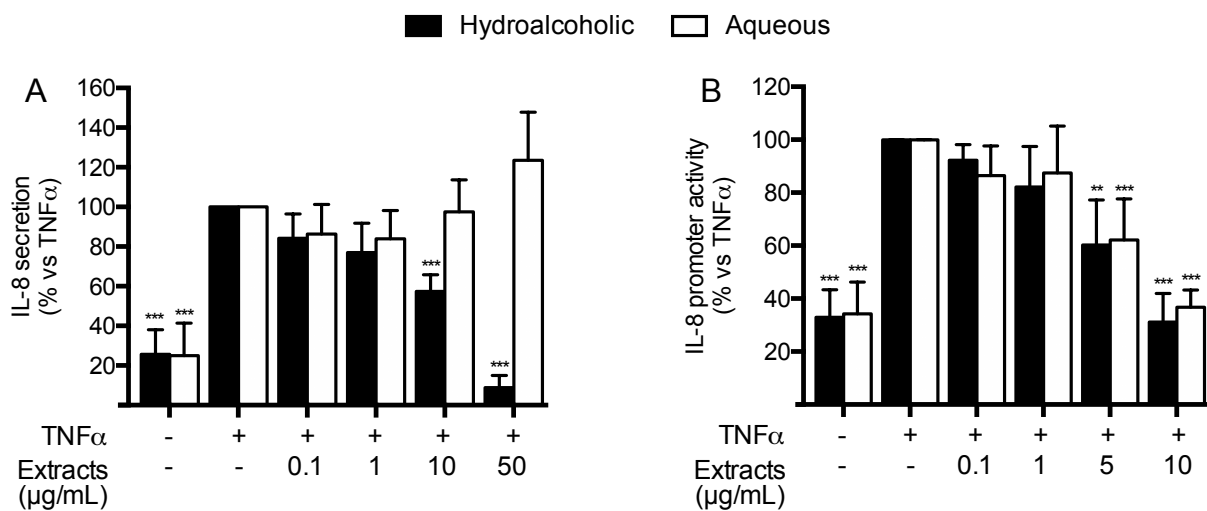
## 3. Results and Discussion

Dried inflorescence powder of *Humulus lupulus* cv. Cascade was extracted with two different solvent mixtures, water (aqueous extract) and ethanol/water (50:50) (hydroalcoholic extract), two solvents suitable for human consumption, and the yields of extraction were respectively 19.66% and 29.96%. Both the extracts preliminarily revealed lack of cytotoxicity on the gastric cell model (AGS), till the maximal concentration tested of 100  $\mu\text{g}/\text{mL}$  (data shown in supplementary materials, Figure S1).

In order to evaluate the potential anti-inflammatory effects of hops, cells were challenged with TNF $\alpha$  in the presence of different concentrations of extract. As shown in Figure 2A, the hydroalcoholic

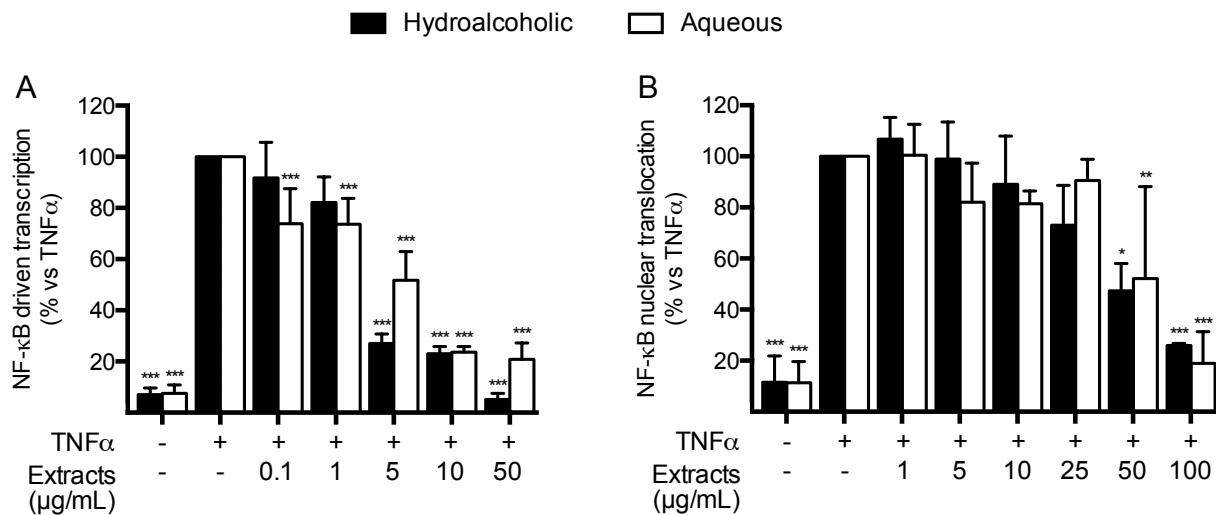
extract inhibited the release of IL-8 in a concentration dependent manner, with an  $IC_{50}$  of 3.95  $\mu\text{g/mL}$ , while the aqueous extract did not show any inhibitory effect till the highest concentration tested of 50  $\mu\text{g/mL}$ . On the contrary, when a reporter plasmid containing the IL-8 promoter region was used, both the extracts were active, completely inhibiting the effect of  $\text{TNF}\alpha$  at 10  $\mu\text{g/mL}$ .  $IC_{50}$  values revealed that the hydroalcoholic extract was slightly more active compared to the aqueous one (2.34 and 3.59  $\mu\text{g/mL}$ , respectively) and this effect was obtained in the same range of the protein release inhibition (Figure 2B).

To investigate the molecular mechanism underlying the effects observed on IL-8, we investigated the ability of hop extracts to impair the NF- $\kappa\text{B}$  pathway. This transcription factor plays a central role in regulating IL-8 gene in several cell models, including gastric epithelial cells. Hop extracts reduced the transcription of the luciferase gene controlled by NF- $\kappa\text{B}$ -LUC plasmid, which contains a promoter region strictly dependent on the NF- $\kappa\text{B}$ , and the effect was obtained in a range of concentrations comparable to those described with IL-8-LUC. Hydroalcoholic extract reduced the transcription with  $IC_{50}$  of 2.44  $\mu\text{g/mL}$ , similarly to the aqueous extract ( $IC_{50}$  2.25  $\mu\text{g/mL}$ ) as shown in Figure 3A. Surprisingly, the effect of the extracts on the inhibition of the NF- $\kappa\text{B}$  nuclear translocation, a former step in the inflammatory activation, required higher concentrations to be achieved.



**Figure 2.** Inhibitory effect of the hydroalcoholic (black bars) and aqueous (white bars) extracts on IL-8 release induced by  $\text{TNF}\alpha$  in gastric epithelial cells (A), in comparison to their effect on IL-8 promoter activity (B), measured by the use of a reporter plasmid regulated by IL-8 promoter region. Results are expressed as percentage of the  $\text{TNF}\alpha$  stimulated condition. EGCG (10  $\mu\text{M}$ ) was used as the reference inhibitor (50% inhibition of IL-8 secretion and 15% for IL-8 promoter activity).

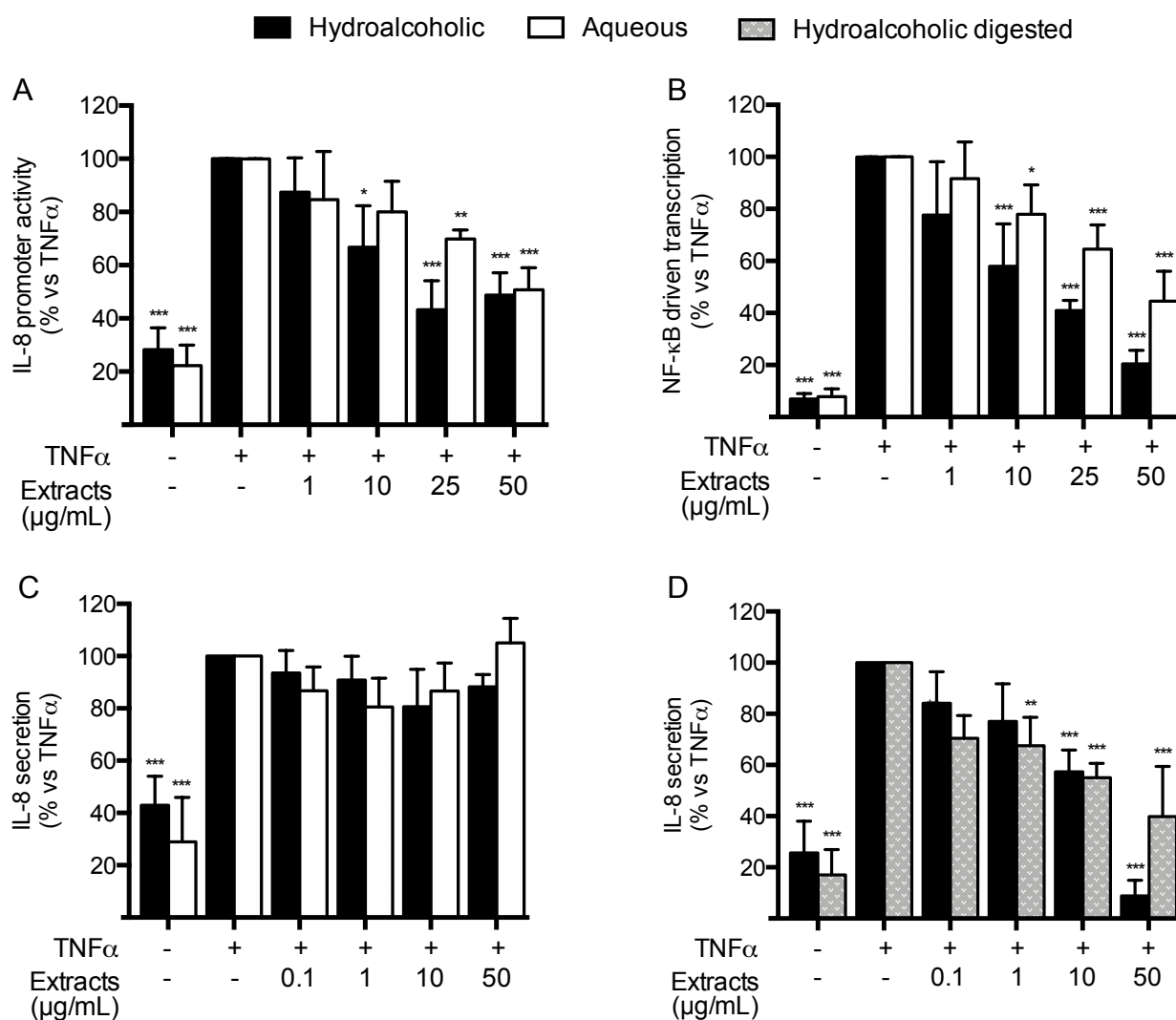
As depicted in Figure 3B, only the highest concentration tested (100  $\mu\text{g}/\text{mL}$ ) brought the amount of nuclear NF- $\kappa\text{B}$  close to the level of the unstimulated control. In this case, both the extracts required more than ten times higher concentrations to impair NF- $\kappa\text{B}$  translocation, compared to the inhibitory effect shown on NF- $\kappa\text{B}$  driven transcription, resulting in  $\text{IC}_{50\text{s}}$  of 33.88 and 42.32  $\mu\text{g}/\text{mL}$ , respectively for hydroalcoholic and aqueous extracts.



**Figure 3.** Inhibitory effect of the hydroalcoholic (black bars) and aqueous (white bars) extracts from hop on NF- $\kappa\text{B}$  driven transcription (A) and their effect on NF- $\kappa\text{B}$  nuclear translocation (B), both induced by TNF $\alpha$  in gastric epithelial cells. Results are expressed as percentage of the TNF $\alpha$  stimulated condition. EGCG (10  $\mu\text{M}$ ) was used as the reference inhibitor (75% inhibition for NF- $\kappa\text{B}$ -LUC driven transcription and 85% for nuclear translocation).

These results suggest that hop extracts have the potential to inhibit NF- $\kappa\text{B}$  mostly by affecting the gene transcription mediated by this factor.

Even if the effect of the extracts was comparable when administered simultaneously to TNF $\alpha$ , except for IL-8 secretion, the situation appeared different when extracts were used as pre-treatment. Although both generally less effective, hydroalcoholic extract applied for 1 hour before the inflammatory stimulus was more active in reducing the IL-8 promoter activity (Figure 4A) and the NF- $\kappa\text{B}$  driven transcription (Figure 4B) ( $\text{IC}_{50\text{s}}$  of 9.60  $\mu\text{g}/\text{mL}$  and 8.11  $\mu\text{g}/\text{mL}$ , respectively) in comparison to the aqueous one ( $\text{IC}_{50\text{s}}$  of 34.91  $\mu\text{g}/\text{mL}$  and 36.82  $\mu\text{g}/\text{mL}$ , respectively). However, one-hour pre-treatment with the hydroalcoholic extract did not inhibit IL-8 release, even at the maximal concentration tested (50  $\mu\text{g}/\text{mL}$ ) as shown in Figure 4C. As expected, the aqueous extract was inactive as well.

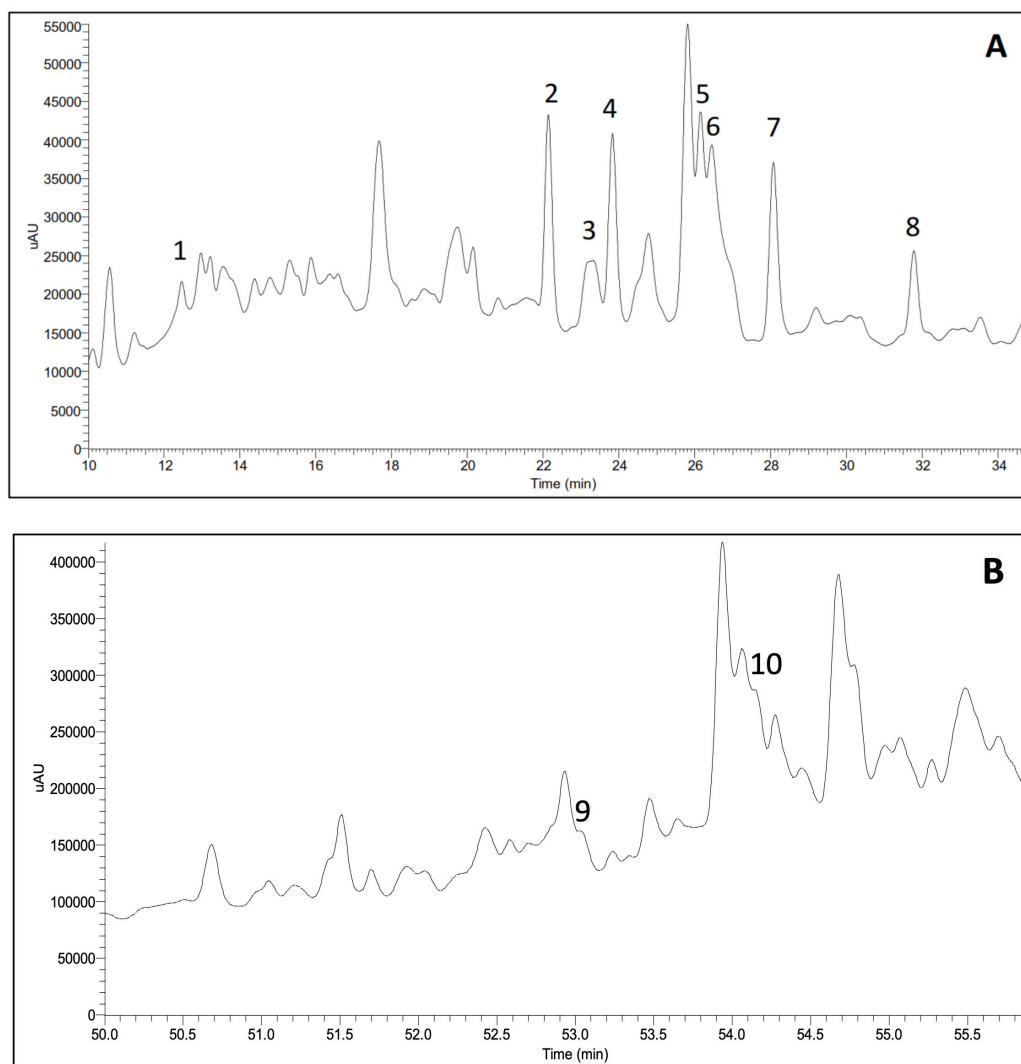


**Figure 4.** Inhibitory effect of one-hour pretreatment of hydroalcoholic (black bars) and aqueous (white bars) extracts from hop on IL-8 promoter activity (A), NF-κB driven transcription (B) and IL-8 secretion (C), induced by TNFα in gastric epithelial cells. Effect on IL-8 secretion of the simulated gastric digestion protocol applied to hydroalcoholic extract (grey bars) in comparison with the non-digested hydroalcoholic extract (black bar), measured during TNFα co-treatment (D). Results are expressed as percentage of the TNFα stimulated condition. EGCG (10 µM), one-hour pretreatment, was used as the reference inhibitor (42% inhibition for IL-8 secretion, 50% inhibition for NF-κB-LUC driven transcription and 18% for IL-8 promoter activity).

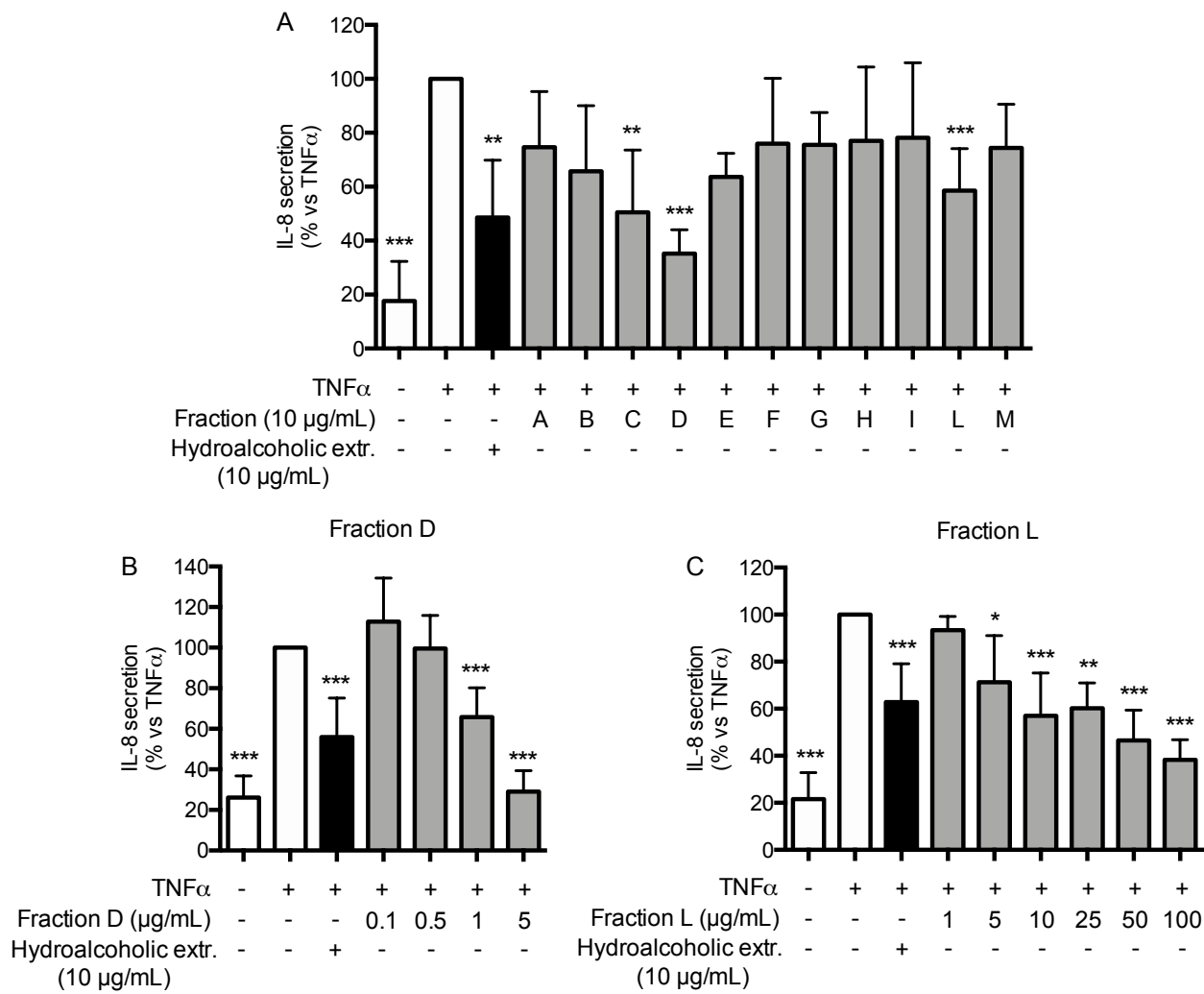
On the basis of the previous results, the hydroalcoholic extract resulted the most promising for the anti-inflammatory activity at gastric level and it was selected for further investigations. Since gastric environment may alter the bioactivity of compounds occurring in the extract, due to low pH and the presence of digestive enzymes, we investigated the effect of an *in vitro* simulated gastric digestion

on the bioactivity of the hydroalcoholic extract. The digested extract was then evaluated on IL-8 release inhibition, as the final step of the inflammatory cascade. As evident in Figure 4D, the simulated gastric digestion showed only slight alteration of the activity of the hydroalcoholic extract, increasing the IC<sub>50</sub> of IL-8 inhibition from 3.95 µg/mL to 6.40 µg/mL. This result strengthens the possibility of using the hydroalcoholic extract from cv. Cascade hop as anti-inflammatory agent at gastric level.

As expected, the hydroalcoholic extract is a complex mixture of secondary metabolites (Figure 5A and 5B). According to mass spectral data and data reported in literature (Li and Deinzer 2007; Magalhaes et al. 2010), some major peaks were assigned as follows: 1) Procyanidin (epicatechin/catechin); 2) Quercetin-3-O-rutinoside; 3) Apigenin-6,8-di-C-glucoside; 4) Quercetin-3-O-hexoside; 5) Kaempferol-3-O-rutinoside; 6) Quercetin-3-O-(6''-malonyl) glucoside; 7) Kaempferol-3-O-hexoside; 8) Kaempferol-3-O-(6''-malonyl) glucoside; 9) Xanthohumol D; 10) Xanthohumol A. In particular, xanthohumol A and xanthohumol D corresponded to 1.86% and 0.28% of the hydroalcoholic extract.



**Figure 5.** HPLC-UV profile of the hydroalcoholic extract: flavonoid (A) and prenylflavonoid region (B). According to mass spectral data and data reported in literature, peaks were assigned as follows: 1) Procyanidin (epicatechin/catechin); 2) Quercetin-3-O-rutinoside; 3) Apigenin-6,8-di-C-glucoside; 4) Quercetin-3-O-hexoside; 5) Kaempferol-3-O-rutinoside; 6) Quercetin-3-O-(6''-malonyl) glucoside; 7) Kaempferol-3-O-hexoside; 8) Kaempferol-3-O-(6''-malonyl) glucoside; 9) Xanthohumol D; 10) Xanthohumol A.

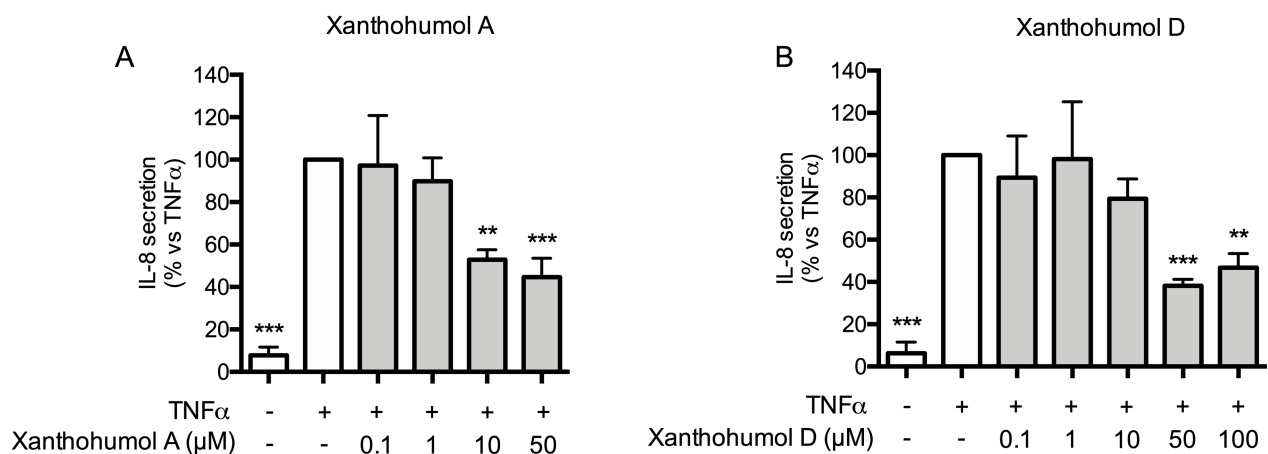


**Figure 6.** Inhibitory effect of the 11 fractions of the hydroalcoholic extract from hop screened at 10 µg/mL on IL-8 secretion induced by TNFα in gastric epithelial cells (A). Evaluation of the effect of fraction D (B) and fraction L (C) in the same experimental setting. Results are expressed as percentage of the TNFα stimulated condition. **The hydroalcoholic extract (10 µg/mL) was used as positive control.**

To identify the bioactive compounds, the total hydroalcoholic extract was fractionated by silica gel column chromatography affording 11 fractions, which were tested for their ability to impair IL-8 release. The analysis on the fractions revealed that only three fractions, C, D and, in much lesser extent, L, significantly inhibited the secretion (Figure 6A). Considering that fraction C and D, given their consecutive elution, most likely contain similar compounds, we tested the effect of fraction D, the most active, and L at different concentrations in the same model. Fraction D (Figure 6B), in agreement with preliminary screening, showed higher effect compared to fraction L (Figure 6C) with

an IC<sub>50</sub> more than six times lower (IC<sub>50</sub> of 1.93 µg/mL and 12.41 µg/mL, respectively). HPLC purification of fraction D revealed that this fraction was mainly composed by xanthohumol A, but it also contained minor amounts (about 10%) of xanthohumol D. **These compounds were identified on the basis of the comparison of their <sup>1</sup>H NMR spectra with those reported in the literature (Kupse & Erhardt, 2007; Nookandeh et al., 2004).**

These molecules were **purified and** separately evaluated on IL-8 secretion (Figure 7A and 7B) and both were able to reduce this parameter, but not to completely inhibit the secretion even at the highest concentration tested of 50 µM. Considering 50 µg/mL of the hydroalcoholic extract, only xanthohumol A reached a concentration of micromolar level, making this molecule capable of contributing to the biological activity. These results suggest otherwise that other molecules could participate in the biological activity of the extract and we cannot exclude that very minor components of the fraction D could significantly contribute to its activity. **Hop prenylflavonoids are efficiently extracted by hydroalcoholic solutions (Biendl M 2013), thus explaining why hydroalcoholic are more active than aqueous extracts.**



**Figure 7.** A: Inhibitory effect of the xanthohumol A (A) in comparison to xanthohumol D (B) on IL-8 secretion induced by TNFα in gastric epithelial cells. Results are expressed as percentage of the TNFα stimulated condition. **EGCG (10 µM) and curcumin (10 µM) were used as reference inhibitors of IL-8 secretion (50% and 81% inhibition, respectively).**

To the best of our knowledge, this is the first study demonstrating the anti-inflammatory activity of xanthohumol D in human gastric epithelial cells. Many literature studies have investigated the biological properties of xanthohumol A, which is mostly known for its anti-proliferative activity in different cancer cell lines, including gastric cells (Wei et al., 2018).



According to the literature, the effect of xanthohumol A on IL-8 was investigated in non-gastric cell lines, such as pancreatic adenocarcinoma cells (Saito et al., 2018) and hepatic carcinoma cells (Dorn, Weiss, Heilmann, & Hellerbrand, 2010), and in both cases the effect was partially mediated by NF- $\kappa$ B inhibition. The different concentrations of the hydroalcoholic extract required to inhibit NF- $\kappa$ B and IL-8 could be explained by the multilevel control mechanism of this chemokine. IL-8 gene in human cells is controlled by different transcription factors such as NF- $\kappa$ B, NF-IL6, nuclear factor- $\kappa$ B repressing factor (NRF) and Activator protein 1 (AP-1) (Jundi & Greene, 2015; Matsusaka et al., 1993; K. Yasumoto et al., 1992). Furthermore, IL-8 mRNA is stabilized by the activity of p38 mitogen-activated protein kinase (p38 MAPK) (Jundi & Greene, 2015). Activation of NF- $\kappa$ B is known to be regulated by reactive oxygen species (ROS) and xanthohumol A may act in opposite directions in this context. In fact this molecule causes *in vitro* the overproduction of ROS, targeting the mitochondrial complex I, a mechanism which is involved in apoptosis of cancer cells (Zhang, Chu, Wei, Liu, & Wei, 2015), but it also induces the transcription of cellular antioxidant enzymes through the modulation of nuclear factor (erythroid-derived 2)-like 2 (NRF2), in different cellular models (Krajka-Kuzniak, Paluszczak, & Baer-Dubowska, 2013; Lee et al., 2011; Yao, Zhang, Ge, Peng, & Fang, 2015).

## Conclusion

Despite the mechanism of action has not been fully clarified, the hydroalcoholic extract investigated in the present study, in particular fraction D, showed a positive effect in the downregulation of IL-8 secretion, **in particular acting on the NF- $\kappa$ B pathway**. This effect could be partially explained by the prenylated chalcones content, especially xanthohumol A and xanthohumol D, however the contribution of other molecules to the biological activity cannot be excluded.

**Our findings demonstrate that** the hydroalcoholic extract of cv. Cascade hop may potentially inhibit the inflammatory reaction at gastric level, although further investigations are needed in order to better clarify **contribution of other compounds in the biological activity** and their mechanism of action.

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## Declaration of interest

Declarations of interest: none.

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