

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



Enrico Sangiovanni^{a,*}, Marco Fumagalli^a, Laura Santagostini^b, Martino Forino^c, Stefano Piazza^a, Elisa Colombo^a, Orazio Taglialatela-Scafati^d, Gelsomina Fico^{e,f}, Mario Dell'Agli^a

^a Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Via Balzaretti 9, 20133 Milan, Italy

^b Department of Chemistry, Università degli Studi di Milano, Via Golgi 19, 20133 Milan, Italy

^c Department of Agricultural Sciences, University of Naples Federico II, Enology Sciences Section, Viale Italia, 83100 Avellino, Italy

^d Department of Pharmacy, University of Naples Federico II, Via Montesano 49, 80131 Naples, Italy

^e Department of Pharmaceutical Sciences, Università degli Studi di Milano, Via Mangiagalli 25, 20133 Milan, Italy

^f Botanic Garden GE Ghirardi, Department of Pharmaceutical Sciences, Università degli Studi di Milano, Via Religione 25, I-25088 Toscolano Maderno, BS, Italy

ARTICLE INFO

Keywords:

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

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 TNFα. 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.

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 TNFα (Crabtree, Shallcross, Heatley, & Wyatt, 1991; Gionchetti et al., 1994; Moss, Legon, Davies, & Calam, 1994; Noach et al., 1994). When exposed to TNFα, gastric epithelial cells release IL-8, a potent chemokine promoting neutrophil infiltration (Crabtree

et al., 1994; Yasumoto et al., 1992a,b; Sharma, Tummuru, Miller, & Blaser, 1995), through activation of NF-κB (Choi et al., 2006; Yasumoto et al., 1992a,b), 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

* Corresponding author.

E-mail address: enrico.sangiovanni@unimi.it (E. Sangiovanni).

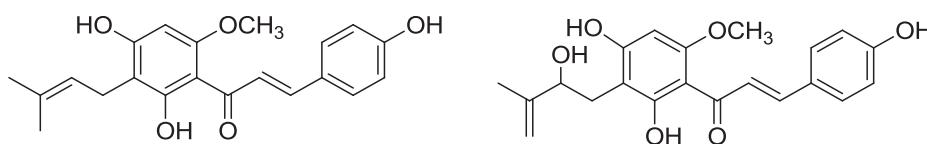


Fig. 1. Chemical structures of xanthohumol A (left) and xanthohumol D (right).

supplements, are considered an interesting source of bioactive compounds, such as xanthohumol A (Fig. 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.

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 α- and β-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). As anti-inflammatory effects, a CO₂ hop extract reduced the prostaglandin E₂ production in adenocarcinoma gastric cells (AGS) by influencing COX-2 enzyme (Tripp, Darland, Lerman, Lukaczer, Bland, & Babish, 2005), while xanthohumol A suppressed the proliferation, apoptosis and metastasis of AGS cells via induction of ROS production and inhibition of the NF-κB 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 cascadone (Forino et al., 2016). In addition, xanthohumol A was found to inhibit the activity of two important pro-inflammatory enzymes, microsomal prostaglandin E₂ 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-κB 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 (Euro-clone 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 TNFα and Human IL-8 Elisa Development Kit were from Peprotech 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 ¹³C Enhanced HCN Cold Probe and by using 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 Hl 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 h 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 g 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 h, 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 H₂O or with 500 mL of EtOH/H₂O (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 μ m column and eluted with H₂O-MeOH (2:8) to obtain pure xanthohumol A (59 mg, 0.20%) and xanthohumol D (6.8 mg, 0.023%) (Fig. 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 \times 4.6 mm, 3 μ m, 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 μ A, 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-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% CO₂.

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 α at 10 ng/mL, was tested after 6 h treatment in the presence of undigested or digested extracts (0.1–50 μ g/mL). EGCG (10 μ 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 μ 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- κ B driven transcription and IL-8 promoter activity, AGS cells were plated in 24-well plates (30,000 cells per well); after 48 h, 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- κ B-LUC, the latter containing the luciferase reporter gene under the control of a NF- κ B responsive promoter (E-selectin promoter). The plasmid NF- κ 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 α 10 ng/mL) and the extract for 6 h. EGCG (10 μ M) was used as the reference inhibitor (75% inhibition for NF- κ 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., Walthman, 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- κ B nuclear translocation

For the NF- κ B (p65) nuclear translocation assay, AGS cells were plated at the concentration of 1.5 \times 10⁶ cells/ml in 60-mm plates. After 48 h, cells were treated for 1 h with the inflammatory mediator (TNF α 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 °C until assayed. The same quantity of total nuclear proteins, measured by Bradford assay, was used to assess NF- κ B nuclear translocation using the NF- κ B (p65) transcription factor assay kit (Cayman), followed by spectroscopy analysis (signal read 450 nm, 0.1 s). Data were expressed considering 100% of the absorbance related to the cytokine-induced NF- κ B nuclear translocation. EGCG (10 μ M) was used as the reference inhibitor (85% inhibition for NF- κ B nuclear translocation).

2.10. Simulated *in vitro* gastric digestion

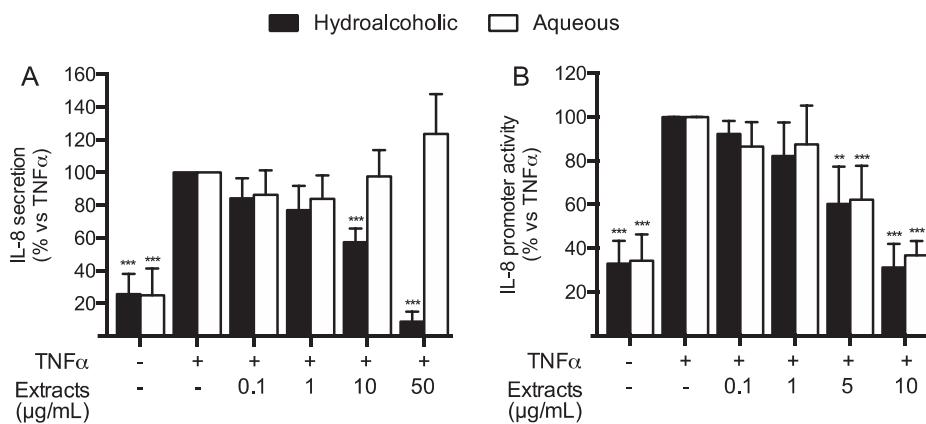
According to a well-established protocol, the gastric digestion was simulated using an *in vitro* approach previously described (Sangiovanni et al., 2015). Briefly, the extract (100 mg) was incubated for 5 min at 37 °C with 6 mL of saliva juice, then 12 mL gastric juice were added to the suspension and the sample was incubated for 2 h at 37 °C. At the end of the incubation, the digested sample was centrifuged for 5 min at 3000 g and the supernatant frozen and lyophilized. All the samples, at different steps, were then stored at –20 °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 α), considering * p < 0.05; ** p < 0.01 and *** p < 0.001. The value of p < 0.05 was considered statistically significant. IC₅₀ 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 μg/mL (data shown in Supplementary materials, Fig. S1).

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jff.2019.03.041>.

In order to evaluate the potential anti-inflammatory effects of hops, cells were challenged with TNF α in the presence of different concentrations of extract. As shown in Fig. 2A, the hydroalcoholic extract inhibited the release of IL-8 in a concentration dependent manner, with an IC₅₀ of 3.95 μg/mL, while the aqueous extract did not show any inhibitory effect till the highest concentration tested of 50 μg/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 TNF α at 10 μg/mL. IC₅₀ values revealed that the hydroalcoholic extract was slightly more active compared to the aqueous one (2.34 and 3.59 μg/mL, respectively) and this effect was obtained in the same range of the protein release inhibition (Fig. 2B).

To investigate the molecular mechanism underlying the effects observed on IL-8, we investigated the ability of hop extracts to impair the NF-κB 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-κB-LUC plasmid, which contains a promoter region strictly dependent on the NF-κB, and the effect was obtained in a range of concentrations comparable to those described with IL-8-LUC.

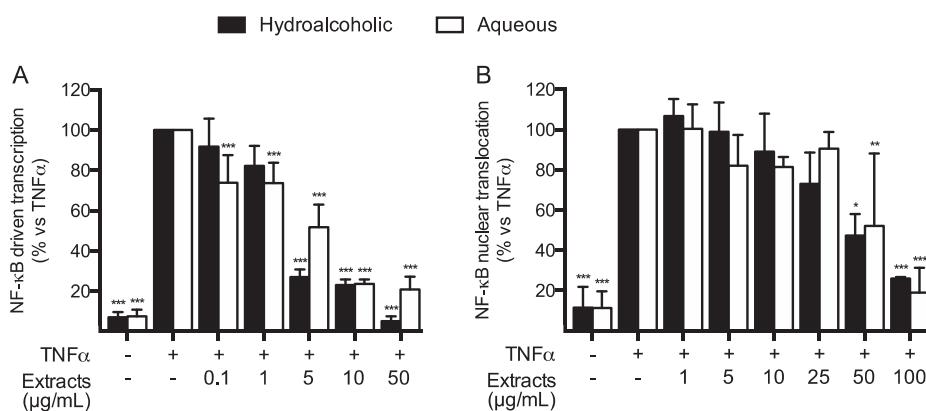


Fig. 2. Inhibitory effect of the hydroalcoholic (black bars) and aqueous (white bars) extracts on IL-8 release induced by TNF α in gastric epithelial cells (A), in comparison to their effect on IL-8 promoter activity (B), measured by the use of a reporter plasmid regulated by IL-8 promoter region. Results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

Hydroalcoholic extract reduced the transcription with IC₅₀ of 2.44 μg/mL, similarly to the aqueous extract (IC₅₀ 2.25 μg/mL) as shown in Fig. 3A. Surprisingly, the effect of the extracts on the inhibition of the NF-κB nuclear translocation, a former step in the inflammatory activation, required higher concentrations to be achieved.

As depicted in Fig. 3B, only the highest concentration tested (100 μg/mL) brought the amount of nuclear NF-κB close to the level of the unstimulated control. In this case, both the extracts required more than ten times higher concentrations to impair NF-κB translocation, compared to the inhibitory effect shown on NF-κB driven transcription, resulting in IC₅₀s of 33.88 and 42.32 μg/mL, respectively for hydroalcoholic and aqueous extracts.

These results suggest that hop extracts have the potential to inhibit NF-κB mostly by affecting the gene transcription mediated by this factor.

Even if the effect of the extracts was comparable when administered simultaneously to TNF α , 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 h before the inflammatory stimulus was more active in reducing the IL-8 promoter activity (Fig. 4A) and the NF-κB driven transcription (Fig. 4B) (IC₅₀s of 9.60 μg/mL and 8.11 μg/mL, respectively) in comparison to the aqueous one (IC₅₀s of 34.91 μg/mL and 36.82 μg/mL, respectively). However, one-hour pre-treatment with the hydroalcoholic extract did not inhibit IL-8 release, even at the maximal concentration tested (50 μg/mL) as shown in Fig. 4C. As expected, the aqueous extract was inactive as well.

On the basis of the previous results, the hydroalcoholic extract

Fig. 3. Inhibitory effect of the hydroalcoholic (black bars) and aqueous (white bars) extracts from hop on NF-κB driven transcription (A) and their effect on NF-κB nuclear translocation (B), both induced by TNF α in gastric epithelial cells. Results are expressed as percentage of the TNF α stimulated condition. EGCG (10 μM) was used as the reference inhibitor (75% inhibition for NF-κB-LUC driven transcription and 85% for nuclear translocation).

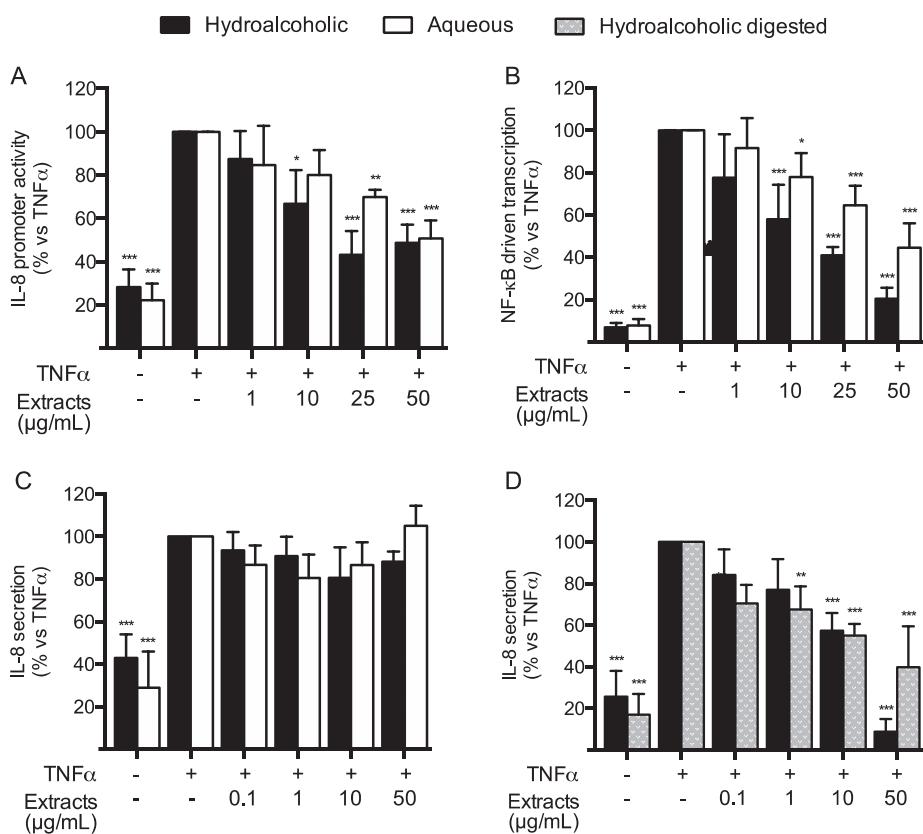


Fig. 4. Inhibitory effect of one-hour pretreatment of hydroalcoholic (black bars) and aqueous (white bars) extracts from hop on IL-8 promoter activity (A), NF-κB driven transcription (B) and IL-8 secretion (C), induced by TNFα in gastric epithelial cells. Effect on IL-8 secretion of the simulated gastric 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).

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 Fig. 4D, the simulated gastric digestion showed only slight alteration of the activity of the hydroalcoholic extract, increasing the IC₅₀ 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 (Fig. 5A and B). According to mass spectral data and data reported in literature (Li & 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.

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 (Fig. 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 (Fig. 6B), in agreement with preliminary screening, showed higher effect compared to fraction L (Fig. 6C) with an IC₅₀ more than six times lower (IC₅₀ 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 ¹H 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 (Fig. 7A and B) 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, 2013), thus explaining why hydroalcoholic are more active than aqueous extracts.

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).

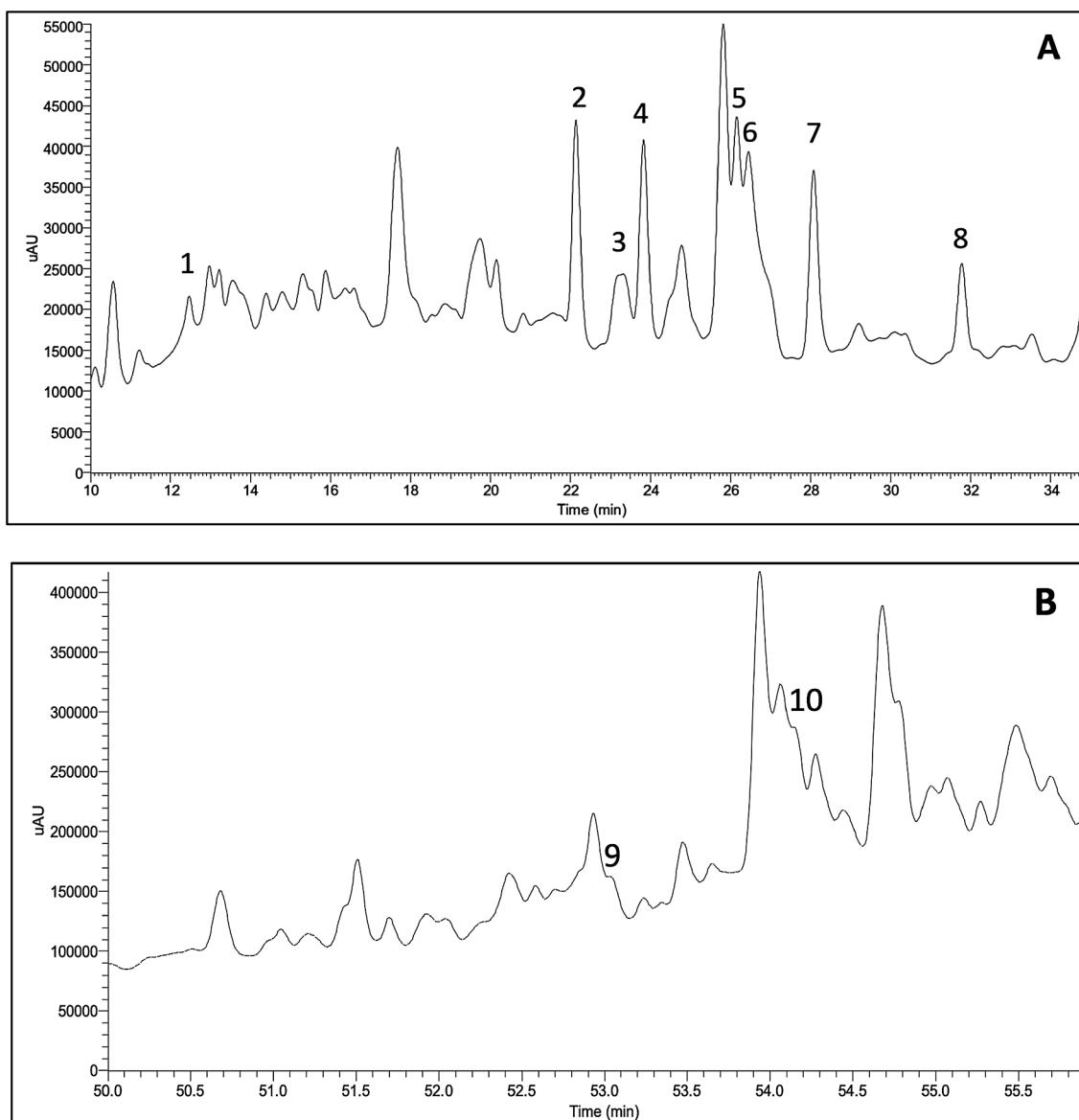


Fig. 5. HPLC-UV profile of the hydroalcoholic extract: flavonoid (A) and prenylflavonoid region (B). According to mass spectral data and data reported in literature, peaks were assigned as follows: (1) Procyanidin (epicatechin/catechin); (2) Quercetin-3-O-rutinoside; (3) Apigenin-6,8-di-C-glucoside; (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.

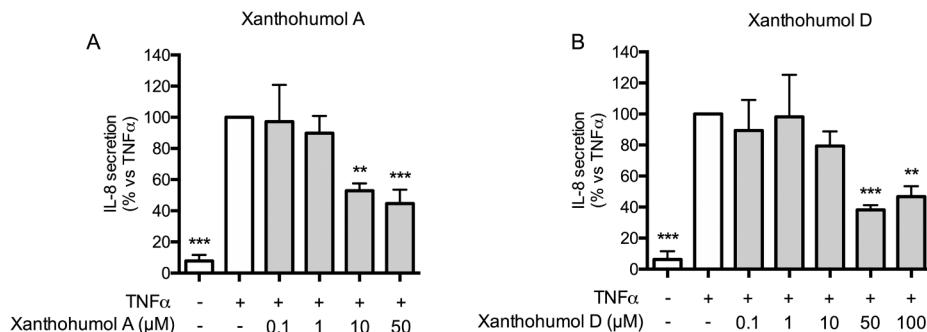
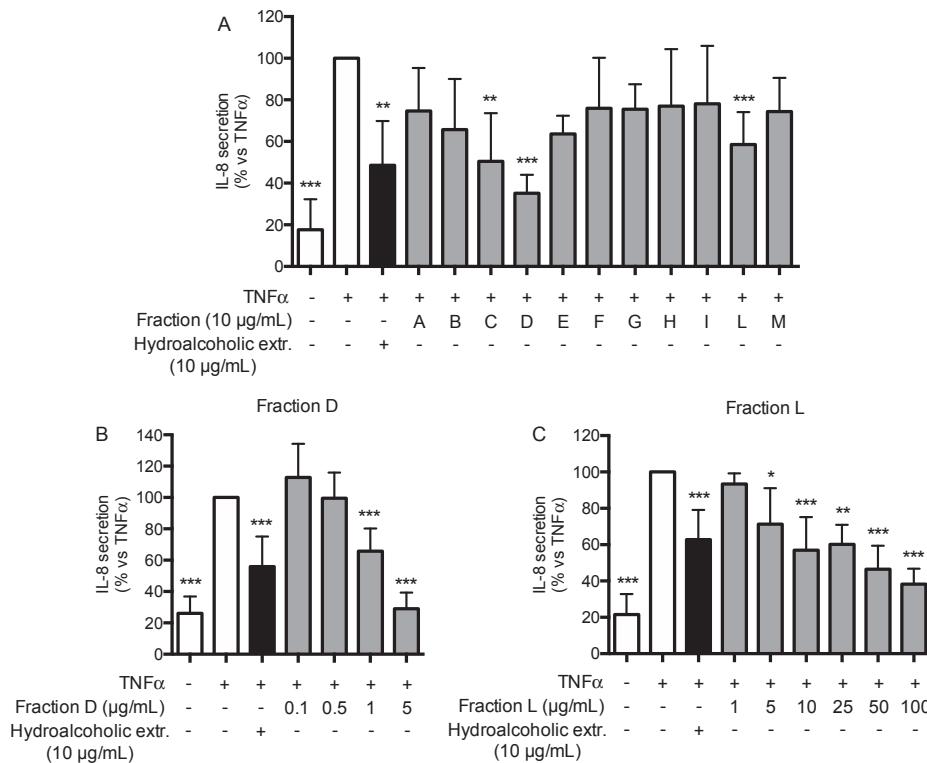
According to the literature, the effect of xanthohumol A on IL-8 was investigated in non-gastric cell lines, such as pancreatic adenocarcinoma cells (Saito et al., 2018) and hepatic carcinoma cells (Dorn, Weiss et al., 2010), and in both cases the effect was partially mediated by NF- κ B inhibition. The different concentrations of the hydroalcoholic extract required to inhibit NF- κ 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- κ B, NF-IL6, nuclear factor- κ B repressing factor (NRF) and Activator protein 1 (AP-1) (Jundi & Greene, 2015; Yasumoto et al., 1992a,b; Matsusaka et al., 1993). Furthermore, IL-8 mRNA is stabilized by the activity of p38 mitogen-activated protein kinase (p38 MAPK) (Jundi & Greene, 2015). Activation of NF- κ 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).

4. 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- κ 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.



5. Ethics statements

The research did not include any human subjects and animal experiments.

Conflicts of interest

All the authors declare no conflict of interests.

Acknowledgments

The Authors would like to thank the farm “La Morosina” (Abbiategrosso, Milan, Italy) for kindly providing the starting hop material, and Dr. T. Shimohata and Prof. A. Takahashi, University of Tokushima, Japan for IL-8 promoter construct. This research was supported by grants from MIUR “Progetto Eccellenza”.

References

- Bartlett, M., & Gilmore, T. D. (1999). Control of apoptosis by Rel/NF-kappaB transcription factors. *Oncogene*, 18(49), 6910–6924. <https://doi.org/10.1038/sj.onc.1203238>.
- Biendl, M. (2013). Isolation of Prenylflavonoids from Hops. *Acta Horticulturae*, 1010, 131–140. <https://doi.org/10.17660/ActaHortic.2013.1010.15>.
- Bonizzi, G., & Karin, M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends in Immunology*, 25(6), 280–288. <https://doi.org/10.1016/j.it.2004.03.008>.
- Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*. <https://doi.org/10.3322/caac.21492>.
- Bulgari, M., Sangiovanni, E., Colombo, E., Maschi, O., Caruso, D., Bosisio, E., et al. (2012). Inhibition of neutrophil elastase and metalloprotease-9 of human adenocarcinoma gastric cells by chamomile (*Matricaria recutita* L.) infusion. *Phytotherapy Research : PTR*, 26, 1817–1822. <https://doi.org/10.1002/ptr.4657>.
- Cermak, P., Palekova, V., Houska, M., Strohalm, J., Novotna, P., Mikyska, A., et al. (2015). Inhibitory effects of fresh hops on helicobacter pylori strains. *Czech Journal of Food Sciences*, 33(4), 302–307. <https://doi.org/10.17221/261-2014-Cjs>.
- Chen, X. Z., Schottker, B., Castro, F. A., Chen, H., Zhang, Y., Holleczek, B., et al. (2016). Association of helicobacter pylori infection and chronic atrophic gastritis with risk of colonic, pancreatic and gastric cancer: A ten-year follow-up of the ESTHER cohort study. *Oncotarget*, 7(13), 17182–17193. <https://doi.org/10.18633/oncotarget.7946>.
- Cho, Y. C., Kim, H. J., Kim, Y. J., Lee, K. Y., Choi, H. J., Lee, I. S., et al. (2008). Differential anti-inflammatory pathway by xanthohumol in IFN-gamma and LPS-activated macrophages. *International Immunopharmacology*, 8(4), 567–573. <https://doi.org/10.1016/j.intimp.2007.12.017>.
- Choi, S. C., Choi, E. J., Oh, H. M., Lee, S., Lee, J. K., Lee, M. S., et al. (2006). DA-9601, a standardized extract of *Artemisia asiatica*, blocks TNF-alpha-induced IL-8 and CCL20 production by inhibiting p38 kinase and NF-kappaB pathways in human gastric epithelial cells. [Research Support, Non-U.S. Gov't]. *World Journal of Gastroenterology*, 12(30), 4850–4858.
- Chuah, S. K., Wu, D. C., Suzuki, H., Goh, K. L., Kao, J., & Ren, J. L. (2014). Peptic ulcer diseases: Genetics, mechanism, and therapies. *Biomed Research International*, 2014,

898349. <https://doi.org/10.1155/2014/898349>.
- Crabtree, J. E. (1996). Gastric mucosal inflammatory responses to Helicobacter pylori. *Alimentary Pharmacology & Therapeutics*, 10(Suppl 1), 29–37.
- Crabtree, J. E., Farmery, S. M., Lindley, I. J., Figura, N., Peichl, P., & Tompkins, D. S. (1994). CagA/cytotoxic strains of Helicobacter pylori and interleukin-8 in gastric epithelial cell lines. *Journal of Clinical Pathology*, 47(10), 945–950.
- Crabtree, J. E., Shallcross, T. M., Heatley, R. V., & Wyatt, J. I. (1991). Mucosal tumour necrosis factor alpha and interleukin-6 in patients with Helicobacter pylori associated gastritis. *Gut*, 32(12), 1473–1477.
- de Gaetano, G., Costanzo, S., Di Castelnuovo, A., Badimon, L., Bejko, D., Alkerwi, A., et al. (2016). Effects of moderate beer consumption on health and disease: A consensus document. *Nutrition, Metabolism & Cardiovascular Diseases*, 26(6), 443–467. <https://doi.org/10.1016/j.numecd.2016.03.007>.
- Dorn, C., Heilmann, J., & Hellerbrand, C. (2012). Xanthohumol suppresses hepatic inflammatory response to acute liver injury. *Journal of Hepatology*, 56 S72–S72.
- Dorn, C., Kraus, B., Motyl, M., Weiss, T. S., Gehrig, M., Scholmerich, J., et al. (2010). Xanthohumol, a chalcon derived from hops, inhibits hepatic inflammation and fibrosis. *Molecular Nutrition & Food Research*, 54, S205–S213. <https://doi.org/10.1002/mnfr.200900314>.
- Dorn, C., Weiss, T. S., Heilmann, J., & Hellerbrand, C. (2010). Xanthohumol, a prenylated chalcone derived from hops, inhibits proliferation, migration and interleukin-8 expression of hepatocellular carcinoma cells. *International Journal of Oncology*, 36(2), 435–441.
- Forino, M., Pace, S., Chianese, G., Santagostini, L., Werner, M., Weinigel, C., et al. (2016). Humidifucol and bioactive prenylated polyphenols from hops (*Humulus lupulus* cv. "Cascade"). *Journal of Natural Products*, 79(3), 590–597. <https://doi.org/10.1021/acs.jnatprod.5b01052>.
- Fumagalli, M., Sangiovanni, E., Vrhovsek, U., Piazza, S., Colombo, E., Gasperotti, M., et al. (2016). Strawberry tannins inhibit IL-8 secretion in a cell model of gastric inflammation. *Pharmacological Research*, 111, 703–712. <https://doi.org/10.1016/j.phrs.2016.07.028>.
- Gionchetti, P., Vaira, D., Campieri, M., Holton, J., Menegatti, M., Belluzzi, A., et al. (1994). Enhanced mucosal interleukin-6 and -8 in Helicobacter pylori-positive dyspeptic patients. *American Journal of Gastroenterology*, 89(6), 883–887.
- Jundi, K., & Greene, C. M. (2015). Transcription of interleukin-8: how altered regulation can affect cystic fibrosis lung disease. *Biomolecules*, 5(3), 1386–1398. <https://doi.org/10.3390/biom5031386>.
- Krajka-Kuzniak, V., Paluszczak, J., & Baer-Dubowska, W. (2013). Xanthohumol induces phase II enzymes via Nrf2 in human hepatocytes in vitro. *Toxicology in Vitro*, 27(1), 149–156. <https://doi.org/10.1016/j.tiv.2012.10.008>.
- Kupse, R. S., & Erhardt, P. W. (2007). Total synthesis of xanthohumol. *Journal of Natural Products*, 70, 1507–1509. <https://doi.org/10.1021/np070158y>.
- Kurasawa, T., Chikaraishi, Y., Naito, A., Toyoda, Y., & Notsu, Y. (2005). Effect of humulus lupulus on gastric secretion in a rat pylorus-ligated model. *Biological & Pharmaceutical Bulletin*, 28(2), 353–357. <https://doi.org/10.1248/bpb.28.353>.
- Lee, I. S., Lim, J., Gal, J., Kang, J. C., Kim, H. J., Kang, B. Y., et al. (2011). Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells. *Neurochemistry International*, 58(2), 153–160. <https://doi.org/10.1016/j.neuint.2010.11.008>.
- Li, H.-J., & Deinzer, M. L. (2007). Tandem mass spectrometry for sequencing proanthocyanidins. *Analytical Chemistry*, 79, 1739–1748. <https://doi.org/10.1021/ac061823v>.
- Magalhaes, P. J., Vieira, J. S., Goncalves, L. M., Pacheco, J. G., Guido, L. F., & Barros, A. A. (2010). Isolation of phenolic compounds from hop extracts using polyvinylpyrrolidone: Characterization by high-performance liquid chromatography-diode array detection-electrospray tandem mass spectrometry. *Journal of Chromatography A*, 1217, 3258–3268. <https://doi.org/10.1016/j.chroma.2009.10.068>.
- Matsusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, K., Kishimoto, T., et al. (1993). Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proceedings of the National Academy of Sciences of the United States of America*, 90(21), 10193–10197.
- Moss, S. F., Legon, S., Davies, J., & Calam, J. (1994). Cytokine gene expression in Helicobacter pylori associated antral gastritis. *Gut*, 35(11), 1567–1570.
- Noach, L. A., Bosma, N. B., Jansen, J., Hoek, F. J., van Deventer, S. J., & Tytgat, G. N. (1994). Mucosal tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8 production in patients with Helicobacter pylori infection. *Scandinavian Journal of Gastroenterology*, 29(5), 425–429.
- Nookandeh, A., Frank, N., Steiner, F., Ellinger, R., Schneider, B., Gerhauser, C., et al. (2004). Xanthohumol metabolites in faeces of rats. *Phytochemistry*, 65, 561–570. <https://doi.org/10.1016/j.phytochem.2003.11.016>.
- Ohata, H., Kitauchi, S., Yoshimura, N., Mugitani, K., Iwane, M., Nakamura, H., et al. (2004). Progression of chronic atrophic gastritis associated with Helicobacter pylori infection increases risk of gastric cancer. *International Journal of Cancer*, 109(1), 138–143. <https://doi.org/10.1002/ijc.11680>.
- Rugge, M., Pennelli, G., Pilozzi, E., Fassan, M., Ingravallo, G., Russo, V. M., et al. (2011). Gastritis: The histology report. *Digestive and Liver Disease: Official Journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver*, 43(Suppl 4), S373–384. [https://doi.org/10.1016/S1590-8658\(11\)00593-8](https://doi.org/10.1016/S1590-8658(11)00593-8).
- Saito, K., Matsuo, Y., Imafuku, H., Okubo, T., Maeda, Y., Sato, T., et al. (2018). Xanthohumol inhibits angiogenesis by suppressing nuclear factor-kappaB activation in pancreatic cancer. *Cancer Science*, 109(1), 132–140. <https://doi.org/10.1111/cas.13441>.
- Sangiovanni, E., Di Lorenzo, C., Colombo, E., Colombo, F., Fumagalli, M., Frigerio, G., Restani, P., & Dell'Agli, M. (2015). The effect of in vitro gastrointestinal digestion on the anti-inflammatory activity of *Vitis vinifera* L. leaves. *Food & Function*, 6(8), 2453–2463. <https://doi.org/10.1039/C5FO00410A>.
- Sharma, S. A., Tummuru, M. K., Miller, G. G., & Blaser, M. J. (1995). Interleukin-8 response of gastric epithelial cell lines to Helicobacter pylori stimulation in vitro. *Infection and Immunity*, 63(5), 1681–1687.
- Shishodia, S., & Aggarwal, B. B. (2004). Nuclear factor-kappaB: A friend or a foe in cancer? *Biochemical Pharmacology*, 68(6), 1071–1080. <https://doi.org/10.1016/j.bcp.2004.04.026>.
- Siregar, G. A., Halim, S., & Sitpu, V. R. (2015). Serum TNF-a, IL-8, VEGF levels in Helicobacter pylori infection and their association with degree of gastritis. *Acta Med Indones*, 47(2), 120–126.
- Tripp, M., Darland, G., Lerman, R., Lukaczer, D., Bland, J., & Babish, J. (2005). Hop and modified hop extracts have potent in vitro anti-inflammatory properties. *Proceedings of the 1st international humulus symposium* (pp. 217–227). <https://doi.org/10.17660/ActaHortic.2005.668.28>.
- Wei, S., Sun, T., Du, J., Zhang, B., Xiang, D., & Li, W. (2018). Xanthohumol, a prenylated flavonoid from Hops, exerts anticancer effects against gastric cancer in vitro. *Oncology Reports*. <https://doi.org/10.3892/or.2018.6723>.
- Xuan, J., Deguchi, R., Yanagi, H., Ozawa, H., Urano, T., Ogawa, Y., et al. (2005). Relationship between gastric mucosal IL-8 levels and histological gastritis in patients with Helicobacter pylori infection. *Tokai Journal of Experimental and Clinical Medicine*, 30(2), 83–88.
- Yahiro, K., Shirasaka, D., Tagashira, M., Wada, A., Morinaga, N., Kuroda, F., et al. (2005). Inhibitory effects of polyphenols on gastric injury by Helicobacter pylori VacA toxin. *Helicobacter*, 10(3), 231–239. <https://doi.org/10.1111/j.1523-5378.2005.00315.x>.
- Yao, J., Zhang, B., Ge, C., Peng, S., & Fang, J. (2015). Xanthohumol, a polyphenol chalcone present in hops, activating Nrf2 enzymes to confer protection against oxidative damage in PC12 cells. *Journal of Agriculture and Food Chemistry*, 63(5), 1521–1531. <https://doi.org/10.1021/jf505075n>.
- Yasumoto, K., Okamoto, S., Mukaida, N., Murakami, S., Mai, M., & Matsushima, K. (1992b). Tumor necrosis factor alpha and interferon gamma synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-kB-like binding sites of the interleukin 8 gene. *The Journal of Biological Chemistry*, 267, 22506–22511.
- Yasumoto, K., Okamoto, S., Mukaida, N., Murakami, S., Mai, M., & Matsushima, K. (1992a). Tumor necrosis factor alpha and interferon gamma synergistically induce interleukin 8 production in a human gastric cancer cell line through acting concurrently on AP-1 and NF-kB-like binding sites of the interleukin 8 gene. *Journal of Biological Chemistry*, 267(31), 22506–22511.
- Zhang, B., Chu, W., Wei, P., Liu, Y., & Wei, T. T. (2015). Xanthohumol induces generation of reactive oxygen species and triggers apoptosis through inhibition of mitochondrial electron transfer chain complex I. *Free Radical Biology and Medicine*, 89, 486–497. <https://doi.org/10.1016/j.freeradbiomed.2015.09.021>.